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Chemical Characteristics of Plant Families of Medicinal Importance. II. Pteridophytes, Gymnosperms and Monocotyledons

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Preparation of this extension of a previous paper on the chemical characteristics of the families of dicotyledons (1) has been prompted by the same motivating factors responsible for the earlier work. There is no place in the modern literature where the interested investigator will find a compilation of chemical compounds which serves to characterize any significant number of plant families or which is potentially useful for this purpose. Consequently, the present summary was undertaken to provide an outline or framework which could serve as guidelines for the further development of the science of chemotaxonomy.

The former paper was limited to a discussion of the dicolytedonous plants of medicinal importance for several reasons. More information is available regarding the chemical composition of such plants, and they are of more direct interest to pharmacognosists. Since the plants covered in this work yield a much smaller number of important drugs than the dicotyledons and constitute a much smaller number of families, less limitation is necessary and coverage has been somewhat more inclusive. All families of pteridophytes, gymnosperms, and monocotyledons, about which chemotaxonomically useful information could be gathered, have been included.

Most of the difficulties encountered by the compiler of chemotaxonomic information stem from two sources: either a lack of information regarding the constituents found in certain taxa, or deficiencies in reporting and indexing such information so as to render it useful in classificational studies. The author hopes that this preliminary survey will help to remedy both these deficiencies by pointing out those areas where further work would be of value and in stimulating phytochemical investigators to consider the systematic significance of their results and to make these known, whenever possible, in their publications.

In the following listing, all references to the number of genera and species in a family are derived, unless otherwise noted, from Lawrence (2).

PTERIDOPHYTES

Equisetaceae: 1 genus of about 25 species.

European investigators have succeeded in isolating alkaloids which are apparently derivatives of lupinane from continental species of the genus *Equisetum*.

Palustrine, together with traces of nicotine, was obtained from *E. arvense* and *E. hiemale*, while *E. palustre* yielded both palustrine and palustridine. Uncharacterized alkaloids have also been reported from *E. telmateia* and *E. sylvaticum*. Interestingly, a recent investigation of some species of *Equisetum* native to England, including *E. arvense*, *E. telmateia*, and *E. sylvaticum*, was unable to establish the presence of alkaloids, other than traces of nicotine in them (3). Whether this was due to variations in environment, the genetic composition of the plants, or to differences in experimental methods has not been elucidated.

Lycopodiaceae: 2 genera and about 100 species.

Alkaloids are widely distributed in the genus *Lycopodium*, having been detected in about a dozen different species and varieties (4). Nicotine occurs in about half of these but because of its relatively simple structure lacks taxonomic significance. The quinolizidine bases (5), lycopodine, acrifoline, annofoline, and annotine are more characteristic; lycopodine, for example, is present in all species native to the Northern Hemisphere which have been examined. The monotypic genus *Phylloglossum* has apparently never been investigated for alkaloids.

Polypodiaceae: About 170 genera and 7000 species.

The ferns have received relatively little chemical attention, and most of the work which has been performed centers around those species possessing anthelmintic properties. Nevertheless, some interesting results have been obtained, and the peculiar, simple to complex, aromatic ketones which have been isolated from species of *Aspidium*, *Athyrium*, *Dryopteris*, and *Polystichum* may be of systematic importance (6). These include such compounds as aspidinol, flavaspidic acid, aspidin, albaspidin, filicic acid, and filixic acid.

Cyanogenetic glycosides, at least some of which are apparently of the amygdalin type, have been reported in twenty-five species representing nine genera of this family (7). On the basis of their occurrence in four species of *Cystopteris*, eight species of *Davallia*, and five species of *Lindsaya* (8), Hegnauer concludes that at least certain genera of the Polypodiaceae may be characterized by cyanogenesis.

GYMNOSPERMS

Cycadaceae: 9 genera and about 100 species.

The toxic glycoside macrozamin has been detected in the seeds of at least eight species of *Macrozamia* and two of *Bowenia* (6, 9). It apparently does not occur in *Macrozamia denisoni*, but this report needs verification. A similar glycoside, cycasin, which apparently differs from macrozamin only in the identity of the sugar moiety has been isolated from *Cycas revoluta*.

These compounds have not been detected in other members of the plant kingdom and would appear to be of systematic importance in at least certain Australian and Japanese genera of this family.

Ginkgoaceae: 1 genus containing a single species.

The dihydric phenol bilobol has apparently been isolated only from the fruit of *Ginkgo biloba* (6), but its structure is so relatively uncomplicated that little classificational importance may be attached to it. No doubt as more plants are analyzed, bilobol will be found elsewhere in the plant kingdom. This has been the case with the corresponding monohydric phenol ginkgol which was initially obtained from *Ginkgo biloba* but has since been identified, in the form of the *trans*-isomer, in caschew nut oil. The same applies to the aliphatic alcohol ginnol, first detected in ginkgo fruit but since isolated from plants belonging to a number of different families.

It would indeed be unusual if this sole surviving representative of its order

did not contain some unique secondary chemical constituent which would set it apart from other plants. On the basis of present evidence it can only be concluded that such a compound remains to be discovered.

Taxaceae: 3 genera and about 13 species.

Hegnauer (10) has detected the complex alkaloidal mixture taxine in the following species and varieties of the genus *Taxus*: *T. baccata* vars. *dovastoniana*, *fastigiata*, *aurea*, and *baccata*; *T. canadensis*; *T. cuspidata* var. *nana*; and *T. media* var. *hatfieldii*. Tyler (11) isolated this alkaloid from *T. brevifolia*.

Taxine thus becomes a significant taxonomic characteristic of the genus *Taxus*, having been found in all species and varieties which have been examined. Although cyanogenetic compounds are also present in some members of the genus, they are absent in others. The alkaloids of the closely related genus *Cephalotaxus*, family Cephalotaxaceae, are not identical with taxine.

Species of the other genera of the Taxaceae, *Torreya* and *Austrotaxus* have apparently not been investigated for alkaloids (12).

Podocarpaceae: 7 genera and about 100 species.

The diterpene phyllocladene has been isolated from the volatile oils obtained from the leaves of several species of the genera *Dacrydium*, *Phyllocladus*, and *Podocarpus* (6). In addition, it has been found in a single species, *Araucaria excelsa*, of the very closely related Araucariaceae and in *Sciadopitys verticillata* of the family Taxodiaceae. An isomer, isophyllocladene, has been obtained from *Cupressus macrocarpa* (13).

In view of these latter occurrences and the fact that some relatively complex terpenes are of widespread occurrence in the plant kingdom, the systematic significance of phyllocladene is difficult to evaluate. Certainly the compound is widespread in the Podocarpaceae, but reports that it, or an isomer of it, exists in several families of the order Coniferae may indicate that phyllocladene is more typical of the entire order than of a single family.

Pinaceae: 9 genera and about 210 species.

Phenolic heartwood constituents appear to possess considerable taxonomic significance (14). All species of *Pinus* which have been investigated, with the exception of *P. lambertiana* and *P. peuce*, contain pinosylvin phenols. In support of Shaw's classification (15), pines of the section Diploxylon almost invariably contain the pinosylvin phenols in combination with the flavanone derivatives pinocembrin and pinobanksin. Pines of the section Haploxylon contain similar constituents plus dihydropinosylvin phenols. The occurrence of these latter compounds is chemotaxonomically useful in characterizing the sections and speaks for the presence in the Haploxylon series of a rather specific enzyme system which is capable of transferring hydrogen from flavanones to the stilbenes. This enzyme system is lacking or incomplete in the Diploxylon pines.

Other genera of the family are less completely investigated. Many species of spruce (*Picea*) contain the lignan conidendrin which also occurs in all hemlock (*Tsuga*) species investigated. Occurrence of the lignan pinoresinol in the "wound healing resin" from *Picea* and *Pinus* and the closely related lariciresinol in *Larix decidua* provides a chemical connection between these three genera. These and the remaining genera of the Pinaceae require intensive chemical investigation before the systematic value of their chemical constituents may be assessed.

Cupressaceae: 15–16 genera and about 140 species.

The chemistry of the constituents of the heartwood of this family has been the subject of intensive investigation by Erdtman and coworkers (14). While it is clear that the members of the Cupressaceae are remarkable terpene specialists

and that relatively few aromatic compounds have been found in the family, the taxonomic value of heartwood constituents is still difficult to judge. In the first place, the chemical survey has not been completed; secondly, many members of the family belong to very small, even monotypic genera. The apparently widespread occurrence of tropolones often in association with phenols such as carvacrol in *Thujopsis*, *Thuja*, *Chamaecyparis*, *Cupressus*, and *Libocedrus* seems to indicate that these genera are intimately related chemically as well as botanically.

Ephedraceae: 1 genus, about 42 species.

Approximately a dozen species of *Ephedra* have been found to contain alkaloids of the ephedrine type (phenylalanine family), but these could not be detected in about six additional species (16). Because of their relatively simple structures these compounds are not of great taxonomic usefulness, ephedrine having been detected in members of the Malvaceae, Papaveraceae, and Ranunculaceae. The single report of ephedrine in *Taxus baccata* (Taxaceae) may derive from the investigation of contaminated plant material. It requires verification.

MONOCOTYLEDONS

Gramineae: About 500 genera and 4000 species (17).

This family is very large, and the taxonomic significance of chemical compounds found in its members is difficult to evaluate because only a relatively few species of commercial significance have been thoroughly investigated. Alkaloids have been detected in ten of sixteen species examined (12) and no doubt exist in a great number of additional species. Commonly, these compounds are rather simple derivatives of phenylalanine or tryptophan (18) and of no special chemotaxonomic significance.

Cyanogenetic compounds have been reported in about ninety-five species representing approximately forty genera, and Hegnauer (7) considers that they may be significant features of certain genera but not the entire family.

Perhaps the characteristic of greatest potential usefulness in the chemical characterization of members of the grass family is the identity of the N-terminal amino acids of their prolamines. Dévényi and Szörényi (19) have established that these N-terminal amino acids are species specific; phenylalanine was identified in wheat gliadin, phenylalanine and glutamic acid in rye gliadin, phenylalanine and threonine in zein, and threonine in avenin. Interestingly, a hybrid of wheat and rye (*Triticale*) yielded a gliadin in which a N-terminal amino acid could not be detected. Likewise, no N-terminal amino acid could be detected in hordein or in the prolamine of *Agropyron christatum*. This type of protein characterization is intriguing, and its extended application in the Gramineae and other plant families is certain to yield results of considerable interest.

Palmae: 210 genera and more than 4000 species.

The seed fats of the members of this family show a remarkable quantitative similarity (20). Although a wide variety of fatty acids exists in them, lauric (45–48 per cent) and myristic (16–20 per cent) are the main component acids. Only a comparatively few species have been subjected to detailed chemical analysis (*Areca catechu*, *Astrocaryum* species, *Attalea* species, *Cocos nucifera*), but the saponification values (240–250) and iodine values (7–30) of other Palmae seed fats indicate that their component acids are probably very similar to those which have been investigated in detail.

Araceae: About 105 genera and 1400–1500 species.

Typical HCN-families are defined by Hegnauer as those in which cyanogenesis has been detected in more than 2 per cent of the species or 10 per cent of the

genera (7). In these families the presence of cyanogenetic compounds appears to have some systematic importance. The Araceae falls in this category, cyanogenesis having been detected in about fifty species representing thirteen different genera.

(Order) Liliiflorae:

The suborder Liliineae is characterized by the widespread occurrence of steroidal sapogenins in the families Liliaceae, Amaryllidaceae, and Dioscoreaceae. An extensive survey of species by Wall and associates (21) has revealed that these compounds occur predominantly in species of *Agave*, *Yucca*, and *Dioscorea*, although other occurrences in the Liliflorae as well as in other orders were noted.

Liliaceae: About 240 genera and 4000 species.

Colchicine has been said to be of systematic significance in this family (22), but the degree of significance was formerly somewhat difficult to evaluate in view of the many erroneous statements in the older literature regarding the occurrence of this alkaloid in plants (23). Fortunately, Šantavý has re-evaluated these old reports using modern scientific techniques and has greatly clarified the knowledge of colchicine distribution (24). He concluded that the alkaloid occurs with certainty in approximately thirty species and varieties of *Colchicum*, *Merendera*, and *Bulbocodium*, as well as in species of the genera *Gloriosa* and *Androcymbium*. It very likely occurs in *Littonia modesta*. Genera previously reported to contain colchicine, which do not, include *Anthericum*, *Asphodelus*, *Chamaelirium*, *Fritillaria*, *Hemerocallis*, *Lloydia*, *Muscari*, *Narthecium*, *Ornithogalum*, *Tofieldia*, *Tulipa*, *Xerophyllum*, and *Zygadenus*.

In view of these findings, the chemotaxonomic significance of colchicine is very much reduced from that attributed to it by Gibbs when, based on erroneous chemical data, he felt the occurrence of the alkaloid strongly supported Hutchinson's revision of the Liliaceae. The compound probably has a limited systematic value for certain tribes of the subfamily Melanthioideae.

Alkaloids of the veratrum type are likewise of limited systematic usefulness. Their occurrence is restricted to the melanthoid genera *Veratrum*, *Schoenocaulon*, *Zygadenus*, and *Fritillaria* (18).

Among the monocots, anthraquinones are found only in the family Liliaceae (*sensu* Krause). Their distribution here is limited to a few genera in the subfamily Asphodeloideae which renders them of little systematic importance (25).

On the basis of chemical information currently available, it is apparent that the Liliaceae is composed of a number of rather diverse chemical types, and no compound or type of compound may be said to characterize the family as a whole.

Amaryllidaceae: 86 genera and about 1310 species.

Alkaloids are characteristic of at least portions of this family; however, they have been detected only in the subfamily Amaryllidoideae (26). This is of particular interest in view of the removal of the alkaloid-free subfamilies Agavoideae, Hypoxidoideae, and Campynematoideae from the Amaryllidaceae in the classification of Hutchinson (27). In view of this, Hegnauer concludes that alkaloids of the phenylalanine family probably occur in all true amaryllidaceous plants (18).

These alkaloids are generally classified as phenanthridine derivatives, and all of them do give rise to this compound or its derivatives, but in some cases this requires drastic experimental conditions. Wildman groups them according to six ring systems and concludes that several more will probably need to be added to complete the classification.

At present there appears to be little relation between given alkaloids or even given ring systems and the various tribes or subtribes of the Amaryllidoideae. All twenty-six genera which have been examined contain lycorine, but the quantity

varies considerably. At the generic level, specific alkaloids are often associated with *Crinum*, *Haemanthus*, *Hymenocallis*, and *Narcissus*, but no such correlation is noted in *Nerine* species.

The agreement between alkaloid distribution and the modern phylogenetic concept of this family is an excellent example of the value of chemistry in systematics.

Dioscoreaceae: 10 genera and about 650 species. More than 600 species in the genus *Dioscorea*.

Aside from the steroidal sapogenins which have been mentioned as occurring in several families of the Liliiflorae, it is difficult at present to ascribe much chemotaxonomic significance to any other type of chemical constituent found in this family. The report of Willaman *et al.* (28) which is supported by that of Wall *et al.* (29) leads to the interesting generalization that alkaloids probably do not occur in *Dioscorea* species in the Western Hemisphere but do occur in some Old World species.

Only a single alkaloid, dioscorine, has been isolated from the tubers of *D. hirsuta* Blume and *D. hispida* Dennst. (30), but it is probable that these plants are conspecific. Positive tests for alkaloids have been obtained in a number of other species (29). Chemical studies have revealed that the alkaloid, a derivative of tropane, is apparently substituted in the 2-position (31).

The extent of the distribution of dioscorine and its importance as a systematic character within the divisions of this family remain to be determined.

Iridaceae: 58 genera and about 1500 species.

The isoflavone glycoside iridin has been isolated from the roots of three species of *Iris* and from *Belamcanda chinensis*, and a closely related isoflavone glycoside tectoridin has been obtained from a fourth species of *Iris* (6). Neither has been reported from other plant families. Although this is too small a sample on which to predicate taxonomic significance, the distribution of these compounds in the Iridaceae is worthy of further investigation.

Zingiberaceae: About 47 genera and 1400 species.

Members of this family are characterized by the presence of volatile oils; the genera *Zingiber*, *Alpinia*, *Curcuma*, *Elettaria*, and *Aframomum* are particularly well-known for their aromatic principles. However, in attempting to select chemical constituents which are representative of the entire family, one is confronted by the same difficulty inherent in applying chemotaxonomic principles to other terpene-rich families, such as the Labiatae (1).

Many terpene derivatives are comparatively uncomplicated biosynthetically and have a widespread distribution in the plant kingdom. Some of those with more complex molecules which may have systematic significance have been identified in only a few species. An example of this type of compound is the monocyclic sesquiterpene hydrocarbon zingiberene which is the main constituent in ginger rhizome oil but which has also been detected in the rhizome oil of *Curcuma zedoaria* (13).

Orchidaceae: About 450 genera and 10,000–15,000 species.

As might be expected, this enormous group of plants has been subjected to little chemical investigation. The difficulties involved in obtaining adequate quantities of authentic materials have prevented detailed examination of all but a few commercially important members of the family.

Loroglossin, a glycoside of undetermined structure, has been found in small quantities in more than a dozen species of *Orchis* and in species of *Ophrys*, *Epipactis*, *Goodyera*, *Bletia*, *Spiranthes*, *Cephalanthera* and *Listera*. The occurrence of the compound is thus restricted to members of the Orchidaceae (32).

Chemical examination of the purified glycoside has revealed that the compound is a monoglycoside with the probable empirical formula of $C_8H_9O_3 \cdot C_6H_{11}O_5$ (33). The presence of loroglossin in plant material may be detected with relative ease by paper chromatography (34), so suitable methods now exist for an exhaustive study of the chemotaxonomic significance of this glycoside in the Orchidaceae.

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Countercurrent Separation of Solanaceous Alkaloids

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In the course of work on the biosynthesis of hyoscyamine and scopolamine in *Datura stramonium* L., it became necessary to effect complete separation of these two alkaloids. Variations in the quantity of total alkaloids per sample (<1 mg to >50 mg) as well as variations in the ratio of the two components during the development of the plant made the utilization of paper chromatographic separation impracticable. A review of the literature revealed several methods for the separation of hyoscyamine and scopolamine by column chromatography. Schill and Agren (1), by means of a series of three buffered kieselguhr columns and a fourth column of aluminum oxide, succeeded in separating these two alkaloids quantitatively but the purity of the products was not reported. Marion and Thomas (2), in their work on the biogenesis of the tropane moiety of these alkaloids, reported that the above method was unreliable and they adopted the method of Evans and Partridge (3). Even this latter method is not without certain disadvantages since there is a considerable variation in the migration of the alkaloids depending on the amount present. Recently, Leete (4) has reported the separation of these alkaloids by a means of celite column.

The distribution coefficients for scopolamine ($K=1$; *p*H 5.48) and hyoscyamine ($K=1$; *p*H 7.0) in a chloroform-phosphate buffer system, reported by Bottomley and Mortimer (5), suggested the possibility of successfully separating the two alkaloids. The following procedure, using a countercurrent distribution apparatus[2], has proved highly efficient in separating mixtures of hyoscyamine and scopolamine obtained from extracts of *D. stramonium*.

EXPERIMENTAL

Preliminary tests to determine the distributions coefficients of hyoscyamine and scopolamine between phosphate buffer and chloroform revealed that when pure alkaloids were employed, the resulting K values (where $K=$concentration of alkaloids in buffer/concentration in chloroform) for scopolamine and hyoscyamine were 0.15 and 2.51 respectively at *p*H 6.8 ± 0.1. With the distribution coefficients known, it was possible to predict the number of the tubes which would contain the maximum amount of the two alkaloids by using the formula [see Gregory and Craig (6)];

$$N = n\frac{K}{K+1}$$

where N is the number of the tube containing the maximum amount of alkaloid with distribution coefficient K and n is the number of transfers (the total number of tubes is $n+1$). Thus for forty-nine transfers, the corresponding values of N for scopolamine and hyoscyamine were 6.4 and 35.0 respectively. Assuming that other substances present in the chloroform extract of the alkaloids from *D. stramonium* would not alter the above distribution coefficients, it appeared that a separation of the two alkaloids could be accomplished using a phosphate buffer, *p*H 6.8 and chloroform.

[1]Based in part, on a Ph.D. dissertation submitted by E. W. Underhill to the Graduate School, University of Rhode Island. Recipient of a National Research Council of Canada Special Scholarship. Present address, College of Pharmacy, University of Saskatchewan, Saskatoon, Canada.

[2]Craig-Post automatic liquid-liquid fractionator.

D. stramonium plants were dried for thirty-six hours at 65°C and the dried material was ground to a number 40 powder by means of a Wiley mill (intermediate model). The powdered material was extracted with ether in a Soxhlet apparatus according to the method described for this drug (7). The volume of the ether extract was reduced approximately 30 ml and this volume was extracted with 10 ml portions of 0.5 N sulfuric acid until complete extraction of the alkaloid from the ether solution was effected. The aqueous solution of the alkaloid salts was made alkaline with ammonium hydroxide and the resulting free bases extracted with chloroform.

Countercurrent separation of the alkaloid material was carried out using equal volumes of the phosphate buffer saturated with chloroform and buffer-saturated chloroform as the two phases. The alkaloids were dissolved in 10 ml of buffer-saturated chloroform and introduced as the lower phase in tube number 0 of the apparatus. At the end of forty-nine transfers, the contents of each tube were made alkaline by the addition of ammonium hydroxide and the tubes were shaken several times. Due to the presence of saponins in the extract, emulsification

TABLE 1. *Countercurrent distribution of hyoscyamine and scopolamine.*

Tube number	Amount[1] of alkaloid	Tube number	Amount of alkaloid	Tube number	Amount of alkaloid	Tube number	Amount of alkaloid
0	—	13	t	26	—	38	+++
1	t	14	—	27	—	39	++
2	t	15	—	28	t	40	++
3	+	16	—	29	t	41	+
4	++	17	—	30	+	42	+
5	+++	18	—	31	++	43	t
6	++++	19	—	32	++	44	—
7	+++	20	—	33	+++	45	—
8	++	21	—	34	+++	46	—
9	++	22	—	35	++++	47	—
10	+	23	—	36	++++	48	—
11	+	24	—	37	+++	49	—
12	t	25	—				

[1]Symbols: —=no alkaloid; +=relative concentration; t = trace.

occurred which required prolonged settling periods of up to 30 minutes before transfer of the upper phase could be carried out. The organic phase of each tube was then removed to individual test tubes and the location of the alkaloids determined using the modified Vitali-Morin colorimetric method reported by Freeman (8).

The above procedure was used for the separation of the alkaloids extracted from twenty-three *D. stramonium* samples, varying from 0.5 g to 30 g. Table 1 shows the relative amount of alkaloid found in the tube series for all these cases. Since the maximum quantity of scopolamine was found in tube 6 (theoretical, tube 6.4), while the maximum for hyoscyamine occurred in tubes 35 and 36 (theoretical, tube 35), it would appear that the distribution coefficients for these alkaloids have not been appreciably altered by other substances present in the original extract.

To verify that the alkaloid occurring in tubes three through eleven was scopolamine while that occurring in tubes thirty through forty-two was hyoscyamine, the contents of each of these tubes were reduced in volume, chromatographed, and compared with alkaloid standards using the paper chromatographic procedure of Drey and Foster (9). The alkaloids were revealed by spraying the air-dried papers with Dragendorff's reagent as modified by Munier and Macheboeuf (10).

The chromatograms, in addition to substantiating the identity of the alkaloids, also revealed the absence of other alkaloid substances in these two fractions.

DISCUSSION

The procedure described for the separation of hyosocyamine and scopolamine is not dependent upon the total amount of the alkaloid nor upon the ratio of the two alkaloids present in the plant extract provided the buffer capacity is sufficient. For the quantities of alkaloid employed in this work a 0.2 M buffer was found to be adequate. Similarly good separations of the principle *D. stramonium* alkaloids can be realized by employing the initial organic extract of the dried plant material prior to the partitioning of the bases into the 0.5 N acid.

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Senecio discolor. The Isolation and Identification of Senecionine and Retrorsine

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It has been suspected that the veno-occlusive disease of the liver seen in Jamaican children has as its origin, the ingestion of "bush teas" containing the extractives of certain toxic plants—one possibly *Senecio* (1). Schoental and Magee (2) demonstrated that subacute and chronic liver lesions could be produced in rats by administering single oral doses of retrorsine, seneciphylline, reddelline (all common to various *Senecio* species) and other alkaloids containing the pyrrolizidine group. More recently, it has been reported (3) that *Senecio discolor*, one of the plants used in the preparation of "bush teas," contains retrorsine and isatidine (N-oxide of retrorsine). Prior to this[1] no description of the alkaloids of *S. discolor* existed.

This research had a twofold purpose; first, to provide a method by which the alkaloids of *S. discolor* could be obtained in the semicrude or purified form for use in pharmacological investigations, and second, to isolate and identify the major alkaloids.

EXPERIMENTAL

Extraction of the Alkaloids.—The dried stems and leaves (4.8 kg) of *Senecio discolor* DC. were ground and extracted for 72 hr with absolute methanol. A modified Soxhlet extractor was used. The extract obtained was concentrated on a steam bath to approximately 3 liters, or one-third of its original volume, and 200 ml of 10 per cent HCl was added. Concentration was continued to remove the remainder of the methanol. The HCl solution was decanted from the tar-like mass which had separated. Additional portions of 10 per cent HCl were used to wash the tar. These acid washings were combined, extracted with chloroform, made alkaline with ammonium hydroxide and the alkaloids removed by repeated extraction with ether. Dragendorff's or Mayer's reagents were used as indicators of the completeness of extraction throughout this work. Upon concentration of the ether, the alkaloids crystallized in a semi-pure form. The yield was 0.86 g.

Because of the low yield obtained in the process of extraction, it was suspected that all the alkaloid material had not been separated. The tar-like material was mixed with approximately an equal volume of Celite 545 and 400 ml of octyl alcohol. The mixture was chilled and the solids removed on a Buchner funnel coated with a thin layer of Celite. The marc, consisting of Celite and the tar components, was thoroughly dried *in vacuo* and pulverized. Both the marc and the octyl alcohol filrate were separately extracted with successive portions of 2 per cent HCl. This extract was concentrated, approximately 5-fold, made alkaline with ammonium hydroxide and the alkaloids were removed by extraction with successive portions of ether. This yielded a larger amount of alkaloids in crystalline form which still contained a significant amount of tan-colored impurities. The yield was 3.84 g.

An additional 1.7 g of alkaloid material was obtained by reworking all fractions according to the same procedure. The total yield of alkaloid material was 6.4 g.

[1]Retrorsine was reported to be present in *Senecio discolor* by Schoental (3) while this research was in progress.

The total alkaloid material was purified by dissolving in chloroform and extracting with 10 per cent HCl. This acid solution was made alkaline with ammonium hydroxide and the alkaloids extracted with ether from which they crystallized upon concentration. The yield of semi-pure alkaloids was 3.47 g.

Recrystallization of the semi-pure alkaloid mixture was carried out from either 95 per cent ethanol or from isopropyl ether : absolute methanol (2:3) with yields of 75 per cent being obtained from the latter solvent mixture.

Paper Chromatography.—All paper chromatograms were obtained using the descending technique. The equilibrating-developing system used was that reported by Adams and Gianturco (4) of equal volumes of 5 per cent acetic acid and n-butanol. The lower phase (5 per cent acetic acid, saturated with n-butanol) was used to equilibrate the Chromatocab while the upper phase (n-butanol, saturated with 5 per cent acetic acid) was used as the developing solvent.

The alkaloids, dissolved in a very small amount of chloroform, were spotted on Whatman No. 1 paper strips (1½ x 24 in.), placed in the Chromatocab and equilibrated for 24 hr. The strips were developed for 18 to 24 hr. After removal and drying, the alkaloids were defined by spraying with Dragendorff's reagent. Two well-defined R_F values were consistently obtained, 0.44 and 0.61.

Separation of the Alkaloids by Partition Chromatography.—Twenty g of silicic acid (Mallinckrodt, chromatographic grade) was thoroughly triturated with 14 ml of 0.5 M, *p*H 5.5 phosphate buffer. The mixture was shaken, in a tightly stoppered flask, with approximately 200 ml of chloroform containing 2 per cent n-butanol which had been previously saturated with the buffer. The tube, 24 x 400 mm, was packed to a depth of 78–79 mm by pouring the slurry and then applying air pressure. In this manner, a column was fashioned which had a flow rate of approximately 1.5 ml/min with a head of 1–6 cm maintained via a siphon arrangement. The sample, 100–500 mg, was applied to the top of the column in a minimum amount (usually 3–4 ml) of chloroform. The most successful developing solvent proved to be the above chloroform—butanol—buffer solution. Five ml fractions were collected, the solvent evaporated and the residue weighed. A representative column prepared in this manner separated the crude or recrystallized material into two alkaloids. The first alkaloid (I), comprising approximately 10 per cent of the total mixture, appeared in fractions 8 to 20; the second (II), comprising approximately 90 per cent of the mixture, appeared in fractions 32 to 180 with a definite peak at fraction 36. Fractions 36 and 180 gave identical infrared spectra.

Identity of the Separated Alkaloids.—Two g of the material recrystallized from isopropyl ether : absolute methanol was separated into the component alkaloids on successive columns and the separated alkaloids combined with their proper counterparts. A recovery of 93–98 per cent was attained.

Senecionine.—Alkaloid I, obtained from fractions 8 to 20, recrystallized twice from absolute ethanol : chloroform (4:1) yielded 110 mg monoclinic plates, mp 242–243°C dec; $[\alpha]_D^{23}$—54.9° (0.0273 g 2.0 ml chloroform); R_F 0.61. The infrared spectra was identical with a known sample of senecionine.

Analysis: $C_{18}H_{25}NO_5$; calculated: C, 64.46; H, 7.51; N, 4.18.
Found: C, 64.58; H, 7.64; N, 4.24.

Retrorsine.—Alkaloid II, obtained from combined fractions 32 to 180, recrystallized from absolute ethanol yielded 1.25 g monoclinic plates, mp 216–217°C dec; $[\alpha]_D^{23}$—53.5° (0.0551 g/2.0 ml chloroform); R_F 0.44.

Analysis: $C_{18}H_{25}NO_6$; calculated: C, 61.52; H, 7.17; N, 3.99.
Found: C, 61.45; H, 7.20; N, 3.96.

SUMMARY

The method used for the isolation of the alkaloids of *Senecio discolor* from the stems and leaves has been described. Paper chromatography indicated the

presence of two major alkaloid components. Separation and purification of the alkaloids has been effected by the use of silicic acid columns buffered at *p*H 5.5. The two alkaloids have been identified as senecionine and retrorsine.

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Indole Derivatives In Certain North American Mushrooms

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A number of indole compounds have been reported to occur in species of basidiomycetes. In some cases, the evidence for the existence of a particular compound in a species is inconclusive, while in other cases occurrence has merely been postulated or suggested on the basis of taxonomic relationship.

Three such examples are the reported occurrence of ergot alkaloids in *Clitocybe subilludens* by Foote, Lauter and Baxter (1), the detection of bufotenine in *Amanita mappa*, *A. muscaria* and *A. pantherina* by Wieland and Motzel (2), and the predicted occurrence of hallucinogenic principles in members of the section Caerulescentes of the genus *Psilocybe* by Singer and Smith (3). During the course of an extensive investigation into the nature of the secondary chemical constituents found in mushrooms, authentic specimens of certain of these species became available, and experiments were carried out to verify and extend the existing information concerning them.

EXAMINATION OF *CLITOCYBE SUBILLUDENS*

In 1953, Murrill (4) reported the isolation of two active principles, one of which "resembled ergot in its effect on the womb," from carpophores and cultured mycelium of this mushroom. A United States patent (1), issued that same year to Foote, Lauter and Baxter, covers "the production of ergot and similar oxytocic alkaloids by cultivation of the mycelium of the fungus *Clitocybe subilludens*."

Two authentic specimens of *C. subilludens* Murr. were employed in this investigation. The first specimen (A) consisted of about one-half of a carpophore of the type collection of this mushroom collected by W. A. Murrill in Florida and generously supplied by Dr. John N. Couch, Department of Botany, University of North Carolina (U.N.C. no. 15172). It is believed that the chromatographic examination of this carpophore reported herein is the first ever conducted on a type specimen of an agaric. The second specimen (B) consisted of a portion of a pileus from a very large carpophore collected near Gainesville, Florida by Dr. L. R. Hesler (collection no. 22959) on December 2, 1957. The specimen had been authenticated by Dr. Erdman West and was furnished through the courtesy of Dr. L. R. Hesler, University of Tenessee.

Specimen A was milled to a no. 60 powder, and 0.55 g was placed in a glass-stoppered, 50-ml Erlenmeyer flask together with 30 ml of petroleum ether, shaken for eighteen hours on a reciprocal shaker, and filtered. The defatted, air-dried marc was then similarly extracted with 15 ml of 70 per cent ethanol for two hours, and the extract, clarified by centrifugation, was evaporated to dryness at room temperature. After redissolving the soluble residue in 2 ml of 70 per cent ethanol, 40-μl quantities of this extract were spotted on sheets of Whatman no. 1 filter paper, together with a number of indole reference compounds. The chromatogram was formed with *n*-propanol:1N ammonia (5:1) and sprayed with *p*-dimethylaminobenzaldehyde as described by Tyler and Malone (5). No ergot alkaloids or other indole derivatives, with the exception of tryptophan, were detected by this procedure.

Failure to detect the ergot alkaloids by the application of this general procedure to specimen A led to the use of a specialized procedure for ergot alkaloids with the larger quantity of material available from specimen B. A 10 g quantity

of finely powdered carpophore was defatted with petroleum ether in a Soxhlet apparatus for twenty hours. The air-dried marc (9.05 g) was then extracted with ammonical ether essentially as described by Silber and Schulze (6), but the extraction time was extended to twenty-four hours. After evaporation of the ether extract at room temperature, the soluble residue was dissolved in 2 ml of 2 per cent tartaric acid solution. Quantities of this extract were chromatographed on Whatman no. 1 filter paper in a wash liquid composed of the upper phase of *n*-butanol: acetic acid: water (4:1:5) and also on paper buffered at *p*H 5, using water-saturated butanol as the solvent (7). A 1 ml quantity was also tested quantitatively for ergot alkaloids with *p*-dimethylaminobenzaldehyde reagent (6). All tests were negative, and it was concluded that the authentic herbarium specimens of *Clitocybe subilludens* which were examined did not contain ergot alkaloids.

BUFOTENINE IN *AMANITA* SPECIES

The hallucinogenic principle of *Amanita muscaria* remains unidentified in spite of numerous attempts to characterize it (8). Considerable interest was aroused by the report of Wieland and Motzel (2) in which the compound "mappin", which had previously been isolated from *Amanita mappa* (Batsch. ex Fr.) Quél. (*Amanita citrina* Schaeff. ex S. F. Gray), was found to be bufotenine. This compound fails to exert psychotropic effects following oral administration but is nevertheless a component in certain native hallucinogens (9). Wieland and Motzel also reported its presence, on the basis of chromatographic evidence, in *Amanita muscaria* (Fr.) S. F. Gray and *A. pantherina* (Fr.) Quél., but this latter occurrence could not be verified by Tyler and Brady (10). Catalfomo and Tyler (11) were also unable to detect traces of the alkaloid in *Amanita muscaria*, *A. gemmata* (Fr.) Gill., *A. calyptroderma* Atkinson & Ballen, *A. aspera* (Fr.) Quél., sensu Ricken, Lange, Rea and Konrad & Maublanc, *A. silvicola* Kaufmann, or in several species of *Vaginata*. Appreciable quantities were found in *Amanita porphyria* (A. & S. ex Fr.) Secr., a species closely related to *A. citrina*. It seemed of value, therefore, to examine other related species of *Amanita* to extend the knowledge concerning the distribution of this compound in the genus.

The following dried specimens, obtained from the indicated sources, were examined.

1. *Amanita tomentella* Kromb.: collected September 13, 1958, St.-Henri, Canada; supplied by Dr. René Pomerleau, Canada Department of Forestry, Forest Biological Laboratory, Sillery, Quebec, P. Q., Canada.
2. *Amanita porphyria* (A. & S. ex Fr.) Secr.: collected September 28, 1960, Lee Forest near Seattle, Washington; authenticated by Dr. D. E. Stuntz, Department of Botany, University of Washington.
3. *Amanita citrina* Schaeff. ex S. F. Gray: collected October 1, 1938 near East Rock, New Haven, Connecticut by D. E. Stuntz (Stz. no. 669).
4. *Amanita brunnescens* Atk.: collected in 1954–1955 in vicinity of Quebec, Canada by René Pomerleau.
5. *Amanita brunnescens* var. *brunnescens*: collected July 13, 1949 near Burt Lake, Michigan by D. E. Stuntz (Stz. no. 5181).
6. *Amanita brunnescens* var. *pallida* Krieger: collected July 24, 1949 near Douglas Lake, Michigan by D. E. Stuntz (Stz. no. 5333).

Each was milled to a no. 60 powder in a Wiley laboratory mill, and 0.25 g quantities of each were placed in small test tubes and shaken with 15 ml of 70 per cent ethanol for approximately twenty-four hours. After centrifugation, the clear supernatant liquids were evaporated to dryness on a steam bath, and each residue was dissolved in 1.0 ml of 70 per cent ethanol. Replicate 40-μl quantities of each were spotted (in 10-μl portions) on sheets of Whatman no. 1 filter paper singly and in combination with suitable reference compounds. After ascending formation for eighteen hours with a wash liquid composed of *n*-propanol:1N ammonia (5:1),

the sheets were dried and treated with *p*-dimethylaminobenzaldehyde or with Pauly's reagent.

Bufotenine was found to be present in *A. tomentella*, *A. porphyria*, and *A. citrina*, although the amount contained in the two former species was considerably greater than in the latter. No trace of the compound was detected in *A. brunnescens* or its varieties.

PSILOCYBIN IN *PSILOCYBE PELLICULOSA*

Singer and Smith (3) have indicated that the majority of the hallucinogenic mushrooms of Mexico probably belong to the section Caerulescentes of the genus *Psilocybe*. They have further suggested that many or all species of the section possess hallucinatory properties, but a recent compilation by Wasson (12) of the known psychotropic mushrooms of this group reveals that some species remain uninvestigated.

Psilocybe pelliculosa A. H. Smith, classified in the stirps Silvatica of the section Caerulescentes, is a small mushroom which occurs on debris and humus in and near conifer forests in California, Oregon, Washington, and Idaho. Its limited habitat coupled with its relatively small size probably account for the lack of attention which it has received, in spite of the widespread interest in psychotropic fungi.

Carpophores of this species, collected on the University of Washington campus in the fall of 1960 and authenticated by A. H. Smith, were dried in a circulating-air oven at 50°C. After milling to a fine powder, 100 mg of the dried material were extracted with 5 ml of cold methanol. Fifty-μl quantities of this extract were spotted on sheets of Whatman no. 1 filter paper and chromatographed by the circular procedure of Rutter (13) in 150-mm petri dishes with water-saturated *n*-butanol (14). The chromatograms were air-dried and sprayed with *p*-dimethylaminobenzaldehyde reagent. This revealed a reddish violet zone with an R_F value of 0.24–0.25 which was identical with reference psilocybin, nor could this spot be separated from that of psilocybin when mixed samples of the two were spotted and chromatographed in this system. Psilocin could not be detected in *P. pelliculosa*, but a second zone which produced a stable light blue color with the *p*-dimethylaminobenzaldehyde reagent was detected at R_F 0.36–0.37. The identity of the compound responsible for this zone remains unknown, but it probably is not a tryptamine or even an indole derivative as evinced by the nature of the color reaction.

SUMMARY AND CONCLUSIONS

Certain North American mushrooms were investigated or reinvestigated for the presence of indole derivatives. The occurrence of ergot alkaloids in *Clitocybe subilludens*, as reported by previous investigators, could not be verified by an examination of two authentic specimens of this species.

Bufotenine was detected chromatographically in samples of *Amanita tomentella*, *A. porphyria* and *A. citrina* but was not present in *A. brunnescens* or its varieties which were examined. These findings point up the chemical diversity which exists within members of the section Phalloideae (Fr.) Quél. of the subgenus Euamanita Lange em. Sing. (15). Species comprising this taxon include *Amanita phalloides* (Vaill. ex Fr.) Secr., *A. virosa* Lam. ex Secr., *A. aestivalis* Sing., and *A. brunnescens* as well as *A. citrina*, *A. porphyria* and *A. tomentella*. Of these, the three latter species have been shown to contain bufotenine, but the compound remains unreported from the other species in spite of an attempt to detect it in *A. brunnescens* in this investigation and the intensive examination of *A. phalloides* by the Wielands (16). A preliminary investigation of *A. aestivalis*, conducted by the author on a very small amount of plant material, gave negative tests for this alkaloid.

It would thus appear that the species of the section Phalloideae may be divided into two groups on the basis of certain secondary chemical constituents; one group characterized by the presence of amanita toxins, the other by the occurrence of bufotenine. Whether morphological or histological characteristics will be found which support this chemical division of the section remains to be determined.

Psilocybe pelliculosa was found to contain psilocybin and must be classified a hallucinogenic mushroom. This finding, which supports the classification of this species in the section Caerulescentes, is the first report of psilocybin in a *Psilocybe* species whose distribution is restricted to the United States.

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Effects of Light Quality on Plant Maturity. I. Duration of Growth, Nutrient Supply, and Photoperiod[1]

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Some reports published on light effects of different wavelengths on plant growth (1,2,3,4) are somewhat inconsistent. This may be due either to differences in photosynthetic activity of various plants, or to differences in light energy or source, or as Wassink and Stolwijk (5) pointed out, to the difficulty in obtaining a narrow band of the spectrum which in turn is of adequate intensity for normal photosynthesis. Other more recent evidence reviewed by Veen and Meijer (6) shows that many differences in plant response are reproducible with better techniques, and that they are often due to inherent differences in plant species.

A few reports (6,7,8) show that fluorescent lamps are fairly satisfactory sources of light for plant growth. Mixtures of light from different kinds of lamps, however, often improve the efficiency of light from a single source (9,10,11). Use of fluorescent lamps for plant growth is increasing, either as total source or as a supplement (12,13,14). While some work has been done with fluorescent light quality effects on vegetative growth (9,11,15), comparatively little is known of such effects on plant maturity and reproduction. Most investigations have dealt with the modifying effects of light quality on photoperiodism (6), and scarcely any with these effects on yields of flowers and fruits. This report is on a continuation of previous work (9,15). It shows the modifying effects of three different factors on mature growth of dwarf pea plants under various fluorescent light qualities and incandescent light of equal intensity. These factors were: (*i*) the duration of total growth in days; (*ii*) the concentration of nutrients; and (*iii*) the daily photoperiod during the mature stage.

MATERIALS AND METHODS

Seeds of pea, *Pisum sativum* L., cultivar Little Marvel, were sown in vermiculite in one-half gallon white square plastic containers. Each container had five small holes for drainage of excess water. The seedlings were thinned to five plants evenly spaced per container. They were allowed to grow under warm white fluorescent light of approximately 800 fc at plant level, and were conditioned to this uniform quality and intensity until blooming, which was about 30 days after sowing. This was under the standard photoperiod described below. Then the plants, at the stage of the first one or two flowers, were subjected to the different light quality treatments for another 30 days, which was regarded as the maturing stage. For any particular test with each light quality there were eight containers of pea plants. During both the early growth period and the experimental light period, the plants were supplied with 80 ml of Hoagland nutrient solution per container twice every week. The plants were watered as necessary. At the end of the various light treatments, all pods more than 1 cm long were collected, and the seeds more than 0.5 cm in diameter were counted and dried at about 50°C for 12 days.

The light equipment was in an air conditioned basement room which provided

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temperatures of 21°C during a 16-hour light period and 16°C during an 8-hour dark period daily. Humidity was not controlled but remained fairly constant. Seven different light qualities were used. These were from six colors (including warm white) of General Electric 48-in fluorescent lamps and from white frosted Champion 100 w incandescent lamps. They were adjusted to provide 300 fc at plant level, with one exception. This exception was the use of warm white fluorescent light at 1500 fc, as well as the lower one of 300 fc, to permit a comparison of effects of two intensities in this quality. These light intensities were the corrected ones obtained by applying the manufacturer's correction factor for the normal luminosity curve (9). These correction factors merely bring the figure for the light intensity measurements into agreement with the values for the luminosity curve of the human eye (16), upon which footcandle measurements are based. They do not correct for the different quantum efficiencies of the various portions of the spectrum. The corrected intensity of 300 fc was the highest obtainable for the red lamps and was limiting for equal intensities. The curves for emission spectra for all of these lamps were similar to those previously published (9,17,18). The light intensities were measured with a General Electric multicell light meter.

The chamber for light quality tests consisted of a frame with black cloth curtains to shield it from adjacent lights. This allowed testing of photoperiods other than the standard one mentioned above. The curtains were so arranged as to allow ample circulation of air and the temperature inside differed little from that of the rest of the room. Inside, there was a rectangular stationary table with four adjustable luminaires (6 lamps each) hanging above. These were separated by white enamel-coated curtains.

RESULTS AND CONCLUSIONS

Three Different Growth Periods.—The growth response of certain plants to light seems to vary with the age and size of the plants (11,19). This experiment was on growth response under the lights with three different mature growth periods. These were 10, 30, and 40 days in duration after the 30-day vegetative period.

The results (table 1) show that pod dry weights and seeds increased significantly between the 10- and the 30-day maturity period, but the increase after the 30-day period was generally not significant. However, the effects of light quality were very pronounced. The mean dry weights of pods and the numbers of seeds were greatest with the high-intensity warm white, less with red, blue, incandescent, pink, and the normal-intensity warm white, in that order, and least with yellow and green light. The differences in yields between the high-intensity warm white and red, the differences among red, blue, and incandescent, and the differences among the normal-intensity warm white, yellow, and green light were statistically not significant.

Three Different Concentrations of Nutrient Solution.—This experiment measured light effects on the mature stage of pea plants as modified by three different concentrations of Hoagland nutrient solution. The nutrient solution was prepared in three different concentrations: double- (x2.0), normal- (x1.0), and half-strength (x0.5), by adjusting the amount of the salts of the macro-nutrients only. An exception was made for these plants to the general nutrient treatments in the vegetative stage. They received only half-strength of the nutrients, *i.e.*, the equivalent of 40 ml of the standard solution at each application. The other three nutrient treatments were begun simultaneously with the start of the light treatments in the mature stage.

The results (table 2) show that the yields of the plants at the three nutrient levels under all light qualities tested did not differ significantly in mean dry weights of pods and numbers of seeds. However, there was again a marked effect of light qualities on these yields. As in the previous experiment, the greatest

mean dry weights of pods and numbers of seeds were obtained with the higher-intensity warm white, next greatest with red and blue, although the difference between the latter two was not statistically significant. The yields were next in size with incandescent, pink, and the normal-intensity warm white, in that order, and least with yellow and green. These results indicate that a higher concentration of nutrients is not an important factor in increasing yields during the mature stage. Many plants, including legumes, seem to absorb the major amounts of their necessary macro- (20) and micro-nutrient elements (21) during the vegetative stage.

TABLE 1. *Effects of different light qualities on pea plant growth in three different growth periods (30-day test period)*

A. Mean dry weight of pods per plant in milligrams

Light		Growth period			Mean
Quality	Intensity	10-day	30-day	40-day	
W. white	300 fc	45.88	87.00	67.75	66.88
Blue	300 fc	114.88	333.75	397.38	281.71
Green	300 fc	2.63	9.00	0.00	3.88
Yellow	300 fc	20.00	0.50	7.00	6.50
Pink	300 fc	51.00	209.75	177.25	146.00
Red	300 fc	136.38	384.38	442.13	320.96
Incand	300 fc	96.38	258.88	282.75	212.67
W. white	1500 fc	157.75	534.88	501.63	398.08
Mean		77.00	227.27	234.48	

L.S.D. (@ .05): Light quality, 123.24; Growth period, 71.67

B. Mean number of seeds per plant

Light		Growth period			Mean
Quality	Intensity	10-day	30-day	40-day	
W. white	300 fc	0.10	0.40	0.53	0.34
Blue	300 fc	0.48	1.15	1.70	1.11
Green	300 fc	0.00	0.03	0.00	0.01
Yellow	300 fc	0.08	0.00	0.05	0.04
Pink	300 fc	0.13	0.90	0.85	0.63
Red	300 fc	0.75	1.50	1.83	1.36
Incand	300 fc	0.35	0.98	1.13	0.92
W. white	1500 fc	0.83	1.95	1.95	1.58
Mean		0.38	0.86	1.00	

L.S.D. (@ .05): Light quality, 0.46; Growth period, 0.27

Three Different Photoperiods.—Another experiment was conducted with shorter and longer photoperiods to determine the effects of such conditions on yields in the maturing stage under the different lights. The light chamber covered with black cloth allowed maintenance of photoperiods of controlled length. The following three different photoperiods were tested: (*i*) 8-hour light-period plus 16-hour dark-period; (*ii*) 16-hour light period plus 8-hour dark-period; and (*iii*) 24-hour light-period without dark-period.

The results (table 3) show a proportionate increase in production of both dry weights of pods and numbers of seeds as the light-period was lengthened, regardless

of the light quality and intensity used. Thus, continuous illumination resulted in the greatest yields, and the 8-hour light-period resulted in the least. Here again, the effect of light quality was very pronounced, and the pattern of plant response was similar to that observed in the previous tests. The mean dry weights of pods and numbers of seeds were greatest with the higher-intensity white light, next greatest with red, blue, pink or incandescent, the normal-intensity warm white, and least with yellow or green, in that order. It has been reported that continuous illumination of tomato plants results in leaf-injury symptoms (22) and

TABLE 2. *Effects of different light qualities on pea plant growth with three different concentrations of nutrient solution (30-day test period)*

A. Mean dry weight of pods per plant in milligrams

Light		Concentration			Mean
Quality	Intensity	x0.5	x1.0	x2.0	
W. white	300 fc	79.63	91.50	76.25	82.46
Blue	300 fc	352.00	365.88	384.75	367.54
Green	300 fc	3.00	2.50	2.13	2.54
Yellow	300 fc	32.38	31.63	42.50	35.50
Pink	300 fc	234.50	196.38	243.50	224.79
Red	300 fc	338.38	378.25	389.50	368.71
Incand	300 fc	271.63	232.50	259.75	254.63
W. white	1500 fc	529.00	507.75	547.38	528.04
Mean		230.06	225.80	243.22	

L.S.D. (@.05): Light quality, 27.43; Concentration, not significant

B. Mean number of seeds per plant

Light		Concentration			Mean
Quality	Intensity	x0.5	x1.0	x2.0	
W. white	300 fc	0.50	0.45	0.45	0.47
Blue	300 fc	1.53	1.68	1.93	1.71
Green	300 fc	0.03	0.03	0.00	0.02
Yellow	300 fc	0.28	0.20	0.35	0.28
Pink	300 fc	1.00	0.78	0.95	0.91
Red	300 fc	1.48	1.73	1.85	1.68
Incand	300 fc	1.05	1.18	1.13	1.12
W. white	1500 fc	2.18	2.13	2.33	2.21
Mean		1.00	1.02	1.12	

L.S.D. (@.05): Light quality, 0.18; Concentration, not significant

a typical chlorosis in the leaves (23), while certain other plants benefit from continuous illumination during growth (24,25). Our results are in general agreement with those reported in the latter citations as regards benefits from continuous light.

All three of these experiments as a whole showed that red light, at equal intensity among all the lamps, was most efficient in producing yields in the mature stage, while the effect of blue light was next in size. Pink and warm white were less efficient. Yellow and green were the least efficient, with green lowest of all. These results were very similar to those obtained for dry weight yields of plants in the vegetative stage (9,26).

A higher intensity of any one of the light qualities tested probably would result in a higher yield, since the higher-intensity warm white light caused production of significantly greater yields than the lower one. The chief reason for this assumption is that white light contains parts of the whole visible spectrum.

TABLE 3. *Effects of different light qualities on pea plant growth with three different photoperiods (30-day test period)*

A. Mean dry weight of pods per plant in milligrams

Light		Photoperiod per day			Mean
Quality	Intensity	8-hr	16-hr	24-hr	
W. white	300 fc	10.13	87.00	255.13	117.42
Blue	300 fc	138.00	333.75	632.13	367.96
Green	300 fc	0.00	9.00	25.50	11.50
Yellow	300 fc	0.00	0.50	101.13	33.88
Pink	300 fc	54.38	209.75	462.25	242.13
Red	300 fc	99.38	384.38	663.63	382.46
Incand	300 fc	49.50	258.88	381.00	229.79
W. white	1500 fc	247.75	534.88	988.75	590.46
Mean		74.89	227.27	438.69	

L.S.D. (@.05): Light quality, 204.85; Photoperiod, 119.34

B. Mean number of seeds per plant

Light		Photoperiod per day			Mean
Quality	Intensity	8-hr	16-hr	24-hr	
W. white	300 fc	0.075	0.400	1.000	0.492
Blue	300 fc	0.600	1.150	2.150	1.300
Green	300 fc	0.000	0.025	0.075	0.033
Yellow	300 fc	0.450	0.000	0.425	0.142
Pink	300 fc	0.475	0.900	1.525	0.958
Red	300 fc	0.475	1.500	2.200	1.392
Incand	300 fc	0.275	0.975	1.275	0.842
W. white	1500 fc	1.225	1.950	3.100	2.092
Mean		0.388	0.863	1.469	

L.S.D. (@.05): Light quality, 0.548; photoperiod, 0.319

SUMMARY

Experiments with a dwarf pea, *Pisum sativum* L., cultivar Little Marvel, were conducted to study effects of various light qualities of equal intensity on dry-weight yields of pods and numbers of seeds per plant, under light-laboratory conditions with three different environmental variations.

For the first of these variations, three different lengths of growth periods during the mature stage showed significant increases under all the lights tested for 30 days as compared to 10 days, but none for 40 days as compared to 30 days. With the second set of variations, that of three different nutrient levels, there were no significant differences in yields. However, with the third set of variations in conditions, increases in length of photoperiod caused marked increases in yields.

Under equal light intensity, with all of the three different environmental variations, the yields obtained were always greatest with red and blue light. Yields

with incandescent, pink, and warm white light were intermediate in size. The poorest was found to be with yellow and green light. A higher intensity of warm white light resulted in much greater yields than the lower one, indicating that a higher intensity of the other lights probably also would increase the yields.

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Viable Species of Algae and Protozoa in the Atmosphere

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Controversy over spontaneous generation and the germ theory of disease stimulated interest in the microbial populations of the air during the mid-nineteenth century. Nearly 100 years ago, pioneer studies on the distribution of microorganisms in the air were made by Pasteur (1), Pouchet (2), and Maddox (3). Charles Darwin (4) noted that a multitude of bodies were carried about by the wind in the form of dust and speculated that few showers fall without leaving some sediment. He stated: "An examination of such sediments or deposits with the microscope will soon materially modify our notions of spontaneous generation, and at the same time show a fertile source from which unexpected hybrid forms may arise." Other researchers like Hudson (5), Beger (6), Huber-Pestalozzi (7), Gislen (8,9), Messikommer (10) have stressed the importance of air currents in the dispersal of microorganisms.

Hudson (5), Gislen (8), and Pennak (11) have indicated that the ability of an organism to form a bouyant spore or cyst will probably also explain the wide distribution of that species.

According to Gislen (9), large numbers of microorganisms are constantly being driven up into the air to return again to earth in rain showers or downward air currents. Microorganisms are very resistant to unfavorable factors found in the air. Their resistance to low temperature, low barometric pressure and drought is superior to that of all other organisms. They are often cosmopolitan, or regionally distributed around the whole globe in certain climatic belts. Some microorganisms are very sensitive to radiations, especially ultraviolet, which seem to restrict their distribution. Under favorable conditions, especially in humid air, the harmful influence of radiation is diminished, and microforms may be transported alive by winds over greater distances than in clear and dry weather.

Meier (12) stated that green algal cells were killed upon a 6-min to 18-hr exposure to ultraviolet beyond 3022 A.

Many other workers continued to probe the qualitative and quantitative makeup of the aerial biota. The National Research Council Report (13) should be consulted for a detailed history of this work.

Most sampling of the aerial biota to date has been concerned with bacteria, fungi, and pollen with only occasional reports of algae and Protozoa in the atmosphere. According to van Overeem (14), no one, as far as he was aware, had attempted to culture algae from the atmosphere. In his atmospheric sampling with aircraft various species of green algae were collected from the 2000 m level. However, there was no indication that these organisms were viable and their classification was not included.

Hymen (15) commented that cysts of Protozoa occur attached to grasses, particles of soil and other objects and may be disseminated by various agents but do not float about in the air to any extent.

Puschkarew (16) estimated that there were about 2.5 viable Protozoan cysts per cubic m of air. He obtained only 13 species, chiefly small amoebae and flagellates and one ciliate (*Colpoda*). By exposing containers of sterile media to the air he found that those exposed in the summer were populated at a much faster rate than those in the winter months. Frequently, sterile cultures exposed to the air failed to develop any Protozoa. He also noted that rain was effective in removing their cysts from the air but did not believe the wind was important in their dispersal.

According to Gregory (17), Miquel, in a study conducted in 1883, estimated about one Protozoan cyst to be present in every 10 m^3 of air.

Other researchers, such as Lackey (18) and Rohnert (19) presented additional indirect evidence of algae in the atmosphere by studying the fauna and flora of water-filled tree holes. Lackey found about 140 species of algae and Protozoa inhabiting such holes. Flagellated forms of algae were predominant, while only a few diatoms were seen and no blue-green algae were recorded.

Gregory, Hamilton and Sreeramula (20) reported that large numbers of *Gloeocapsa* were found in the air at certain times over England.

Maguire (21) studied the biota found in the leaf axils of *Dipsacus sylvestris* (teasel) in New York state. From examination of 113 axils, he noted that 67 per cent contained *Colpoda*, 59 per cent contained *Cyclidium* and 9 per cent contained filamentous algae. The means by which these water-filled axils were colonized was not discovered but was believed to be by wind, birds or insects. Maguire also sampled the air by use of a Millipore filter. The study was not considered successful, he believed, because of desiccation. He stated: "A successful filtering procedure would probably involve the removal of the dissemules from the air by bringing them gently into contact with water, thereby avoiding desiccation and damage from impact."

Meier and Lindberg (22) collected objects on petrolatum-coated slides at altitudes up to 3000 ft off the coast of Greenland and found "various fungal spores and hyphae, apparently some unicellular algae, and pollen grains." The algae, however, were not viable.

Talling (23) observed that the dispersal of small, viable, resting stages of microorganisms in wind-borne dust is frequently postulated but difficult to demonstrate. Some phycologists have emphasized the importance of wind dispersal for fresh-water algae. However, the total number of species obtained has been small. It is probably more appropriate to conclude, as Puschkarew (16) did, that such culture experiments have not established the importance of wind in the dispersal of aquatic micro-fauna and flora. Although examples of the dispersal of small aquatic organisms by the wind have been established, the over-all significance of such dispersal is still not clear.

In a previous study the author (24) exposed finger bowls, 6 in. in diameter, containing 50 ml of sterile pond water, to the air at a height of 6 ft for periods of 45 min to 32 hr. The exposed pond water was then poured into flasks containing steamed soil-water medium. Twenty-three species of algae and 3 species of Protozoa were recorded. The higher aquatic plants found growing in some of these cultures included *Utricularia* sp., *Elodea* sp., as well as moss protonema and fern prothallia. The organisms observed living in the culture, as revealed by 96 examinations of 32 cultures, are given in table 1.

There can be little doubt that certain species of algae and Protozoa can be dispersed by air currents. To what extent they are dispersed and under what specific environmental conditions and altitudes they are transported is not known. A preliminary study was undertaken to sample the atmosphere for viable algae and Protozoa. During a 9-month sampling period from September, 1959, through May, 1960, 15,524 ft^3 of air were drawn through modified impingers and membrane filters. A total of 12,157 ft^3 of air was drawn through modified impingers containing 50 ml of sterile soil-water extract at a rate of 1.0, 0.8, or 0.56 ft^3 per min over a 6-hr sampling period. Membrane filters were employed for sampling 3,367 ft^3 of air at rates of 0.25, 0.37, or 0.49 ft^3 per min over a 3-hr period. One 6-hr sample or two 3-hr samples were collected from 12 midnight–6 AM, 6 AM–12 noon, 12 noon–6 PM, or 6 PM–12 midnight.

MATERIALS AND METHODS

Medium preparation.—Soil-water medium (25) was prepared by placing 17 g of loamy soil (*p*H 6.5) in a 125 ml Erlenmeyer flask. Fifty ml of filtered lake

water was then carefully added and the flasks plugged with cotton and capped with aluminum foil. The flasks were steamed for 1 hr on each of 3 consecutive days. Four of every 24 flasks prepared were retained as control flasks. After a 3-month culturing period, the *p*H of the uninoculated culture medium varied from 6.3 to 7.9 as compared to 5.7 to 6.7 for the inoculated flasks containing algae or Protozoa.

Sampling station and air sampling procedures.—The air sampling station, located in the village of Port Sanilac, Michigan, was situated on a ridge 637 ft west and about 40 ft above the level of Lake Huron. The elevation was approximately 675 ft above sea level.

The wind direction and velocity were indicated by a wind vane and an anemometer located 28 ft above the ground. Observations were made for 5 to 15 min, depending upon the steadiness of the weather conditions every hour during the sampling period. These observations represented the wind conditions for the remainder of the hour. Because this was a preliminary qualitative study, expensive wind-directional and speed-recording equipment were not procured. Sky conditions, snow and rainfall were recorded during the 24 hr prior to the sampling period (table 2) as well as during the sample period (table 3).

A sampling basket was elevated by use of a rope and pulley to heights of 15 or 27 ft above the ground. The sampling basket contained a hygrothermograph and either a modified impinger or a membrane filter sampler. The sampling apparatus was connected to an air pump operated by an electric motor by means of 40 ft of rubber tubing. The volume of air sampled was indicated by a flowmeter which was calibrated and rechecked at the Michigan Department of Health Laboratories, Lansing.

A modification of an "air washer", first described by Rettger (26), was used to collect viable forms of life from the atmosphere. All attempts by the author to sample algae and Protozoa from the atmosphere by the use of impingers at sampling rates of 1 and 2 ft^3 of air per min were unsuccessful. Modified impingers (water bottles) were devised for sampling viable species of algae and Protozoa in the atmosphere. Inexpensive collecting bottles and glass tubing with an internal diameter of 4 mm were used in constructing the collecting apparatus. The orifice of the intake tube in the collecting medium was 6.35 mm above the bottom of the bottle. Upon completion of the sampling, the mouth and neck of the water bottle were flamed over an alcohol lamp and the contents added to culture flasks of soil-water medium.

Early in the study a series of 2 water bottles connected by rubber tubing was employed in the modified impinger sampler. After sampling approximately 6,100 ft^3 of air, the second water bottle was discontinued, because microscopic examinations of the cultures inoculated with the exposed medium revealed no viable algae and Protozoa. Positive findings were always present in the first water bottle in the series.

When used during freezing weather, the water bottles were wrapped with a heating tape and enclosed with aluminum foil. When the air temperature was −2.2°C, the collecting medium in the bottle was 3°C. With an air temperature of 0°C, the collecting medium was 4°C.

A Millipore Filter, AA type, was employed in the membrane filter sampler. Following a 3-hr sampling period, the membrane filter was removed from the holder aseptically and cut in half with sterile shears. One-half was mounted on a slide and covered with one drop of immersion oil. Microscopic examinations were made with a 43x objective and 10x ocular. The remaining piece was placed in a flask of soil-water medium for culturing.

Culturing procedure.—The culture flasks, upon inoculation with approximately 50 ml of exposed soil-water extract or one-half of an exposed membrane filter, were placed in an algal culturing unit for 3 months. Microscopic examinations

TABLE 1. *Algae and Protozoa found in sterile pond water exposed to the air.*

Date 1956	Exposure time	Relative humidity %	Sky condition[a]	Air temp min-max[c]	Max wind velocity mph	Organisms (Genera) obtained
7/12	45 min	75–88	C	16–27	4	Vaucheria sp.
7/13	1 hr		PC	16–26	4	Chlorella sp. Nannocloris bacillaris Pleodorina californica Oscillatoria sp. Euglena sp.
7/17	45 min	66	PC	15–25	4	Euglena sp.
7/25[b]	6 hr	51–63	Cl	15–32	2	Chlamydomonas sp. Chlorella vulgaris Nannochloris bacillaris Oedogonium sp. Sphaerocystis Schroeteri Ulothrix sp. Nostoc sp. Colpoda sp.
7/25	5 hr	72–82	C	19–27	1	Chlorella vulgaris Nannochloris bacillaris Ulothrix sp. Utricularia sp.
8/2	5½ hrs	48–56	Cl	12–25	5	Euglena sp. Gloeocystis sp. Pelogloea bacillifora
8/3	25½ hr	51–87	C	13–25	1	Chlorella sp. Chroococcus sp. Gloeocapsa sp. Euglena sp.
8/6	29 hr	86–90	C	18–27	3	Rhizoclonium sp.
8/15	32 hr	44–90	PC	16–29	2	Gloeocystis sp. Protococcus sp. Rhizoclonium sp. Sphaerocystis Schroeteri Chroococcus sp. Euglena sp.
8/16	4 hr	54–61	PC	18–31	4	Chlorella sp. Oscillatoria sp. Protococcus sp.
8/17	7 hr	59–70	PC	17–31	2	Oscillatoria sp. Unclassified amoeba
8/20	24 hr	53–78	PC	9–21	2	Gloeocystis sp. Lyngbya-like cells Oscillatoria sp. Phormidium sp. Unclassified flagellate

[a] *C*, cloudy; *PC*, partly cloudy; *Cl*, clear.

[b] Cheesecloth netting was placed over the remainder of the fingerbowls to prevent contamination by insects.

[c] Air temperature during exposure.

were made at 1–2, 4–6, and 10–12 weeks. The light intensities in the unit varied from 165–260 ft-c, most cultures receiving 200–260 ft-c from 6 cool white fluorescent bulbs. The cultures received 16 hrs of light and 8 hrs of darkness each day.

The temperature in the algal culturing unit varied with a range of 15–25°C during September through December, 5–18°C during January through April, and 14–24°C during May through July. The average temperature for September-December and May–July was 20°C, while January–April was 14°C.

TABLE 2. *Atmospheric conditions 24 hours prior to sampling.*

Flask no.	Sky conditions[a]	Relative humidity	Air temp range	Wind direction	Ave wind velocity mph	Max gusts mph
19	Cl	47–93%	20–28.9	SW	0–15	15
29	C	56–98%	9.4–16.1	NE–N–NW	0–5	11
42	PC	60–92%	10.5–16.1	N	2–6	16
61	C	50–95%	4.4–11.1	W	0–2	11
68	C	45–98%	−6.6– 3.3	NW	11.5	17
75	C	53–98%	−3.3– 1.7	WNW	0–2	none
83	C	42–92%	2.2– 5.6	WSW	2–4	11
85	PC	42–92%	−3.3– 5.6	WSW	2–4	15
94	PC	72–92%	2.2– 6.1	SW	6–8	18
111	C, snow, 2″ snow cover	56–94%	−3.3– 1.7	S	0–4	10
137	C	50–96%	0–12.2	SW	10–15	36
152	C	22–80%	3.3–16.7	NE	0–2	none

[a] *C*, cloudy; *PC*, partly cloudy; *Cl*, clear.

RESULTS

Viable species of algae and Protozoa were found in the atmosphere under the following conditions: relative humidity, 28–98 per cent; temperature, −2.8 to 28.0°C; wind velocities, 0–2 to 4–15 mph. Seven viable species of algae and Protozoa were obtained as well as bacteria, moss, fern, and fungal spores. Ten cultures contained tufts of moss protonema. *Chlorella vulgaris*, *C. ellipsoidea*, *C.* sp., *Chlorococcum* sp. *Navicula* sp., *Peranema* sp., and one unclassified zooflagellate were collected from the atmosphere under the environmental conditions given in table 3. The identifications were made on living cultures containing both unialgal and mixed populations. No blue-green algae (Cyanophyta) were collected from the atmosphere with these sampling procedures.

The cultures containing algae were retained, a portion of the algae being preserved in Transeau's solution (6 parts water, 3 parts 95 per cent ethanol and 1 part formalin). Mixed and unialgal populations of organisms are growing in the 12 positive flasks in the original culture medium to date. Those cultures which were negative after 5 months of culturing were discarded. The negative data have not been presented, as a total of 153 flasks were inoculated and cultured.

TABLE 3. *Species of viable algae and Protozoa found with sampling data and environmental conditions.*

Flask no.	Date of sample	Time of sample [a]	Height of sample ft	Rate in ft³/min	Method of sampling	Organisms found[b]	Air temp range	Relative humidity	General wind direction	Ave wind velocity mph	Max gusts mph	Barometric pressure	Sky conditions[c]
19	9/23/59	12N–6PM	27	1.0	WB[d]	Chlorella vulgaris	22.8–28.9	43–65%	WSW	4–8	15	30.00 steady	PC
29	10/ 1/59	12M–6AM	27	1.0	WB	Chlorella vulgaris Peranema sp.	14.4–15.6	85–95%	NNW	2–4	14	30.00 steady	C mist
42	10/ 2/59	6PM–12M	27	0.8	WB	Chlorella ellipsoidea	15.6–16.1	88–98%	SSE	2–8	15	30.04–29.90 falling	PC
61	10/13/59	6–8PM	15	0.49	MF	Chlorococcum sp.	3.3– 7.8	46–87%	N	0–2	none	29.98 steady	PC
68	11/20/59	12N–3PM	15	0.25	MF	Chlorella ellipsoidea	0.56– 2.2	48–58%	WSW	0–2	none	30.10–30.12 rising	C
75	11/28/59	12N–6PM	27	0.56	WB	Chlorella ellipsoidea	−1.1–0.56	48–65%	N	4–6	12	29.98 steady	PC
83	12/ 3/59	12N–6PM	27	0.56	WB	Chlorella ellipsoidea	3.3– 8.9	28–70%	SW	4–6	15	29.86–29.84 falling	Cl
85	12/ 3/59	6PM–12M	27	0.56	WB	Chlorella ellipsoidea	1.1– 3.3	70–88%	SSW	2–4	12	39.84 steady	Cl
94	12/16/59	12M–6AM	27	0.56	WB	Chlorella vulgaris Navicula sp., Peranema sp.	0–7.8	56–98%	W	0–6	15	29.70–29.96 rising	Cl
111	2/18/60	12N–6PM	15	0.56	WB	Chlorella sp.	−2.8–2.8	37–65%	NNW	0–4	7	29.78–29.76 falling	PC
137	4/12/60	6AM–9AM	15	0.25	MF	Chlorococcum sp. unclassified zooflagellate	4.4–14.4	30–60%	W	0–4	12	29.72–30.06 rising	Cl
152	5/13/60	9AM–12N	15	0.25	MF	Chlorella ellipsoidea	7.7– 9.4	75–95%	NW	0–2	none	29.82 steady	C, light rain

[a] *N*, noon; *M*, midnight. [b] Taxonomy after Prescott, G. W., (27). [c] *C*, cloudy; *PC*, partly cloudy; *Cl*, clear. [d] *WB*, water bottle; *MF*, membrane filter.

The demonstration of growth under fluctuations in *p*H, temperature, and light intensity in the algal culture unit was obtained. Five flasks of the original culture medium inoculated with lake water containing mixed populations, maintained growth over a 10-month culturing period. A list of the genera of algae and Protozoa cultured under these conditions is given in table 4.

DISCUSSION

Sampling was conducted under extreme environmental conditions, including freezing rain, heavy snow, and fog during the fall, winter and spring.

Short sampling periods (3–6 hr) were expected to produce more uniform and valid results for several reasons. There would generally be less variation in air temperature, relative humidity, and other environmental conditions. The viable cells in the vegetative or spore stage might be destroyed by desiccation during a long sampling period. A change in the chemistry of the collecting medium might occur from the accumulation of the various chemicals, fumes, and other particles present in minute amounts in the air if sampled for long periods. Such accumulation may also be toxic to the organisms on the membrane filter. A slow rate of sampling, usually less than 1 ft^3 per min, was employed to reduce the possibility of damage to the cells.

TABLE 4. *Genera of algae and Protozoa obtained from lake water and cultured over a 5–10 month period under fluctuating environmental conditions in the algal culturing unit.*

Chlorophyta:	*Ankistrodesmus, Characium, Chlamydomonas, Chlorella, Closterium, Gloeocystis, Oedogonium, Oocystis, Pediastrum, Scenedesmus, Spirogyra.*
Chrysophyta:	*Chromulina, Fragillaria, Navicula, Tabellaria.*
Cyanophyta:	*Anabaena, Chroococcus, Nostoc, Oscillatoria, Phormidium, Stigonema.*
Euglenophyta:	*Euglena.*
Protozoa:	*Amoeba, Oikomonas, Peranema, Stylochia*-like ciliate, one unclassified ciliate, two unclassified flagellates.

These concepts are supported by Markee (28). He found that short sampling periods assisted in maintaining uniformity of the values of the meterological parameters with the sampling period. Because of the wide variations in environmental conditions even during 3- and 6-hr intervals, it seems preferable to shorten the sampling period to 1 hr. As shown in table 3, the 3-hr intervals of sampling are more uniform than those of 6-hr.

Fluctuations in cultural conditions usually allow for the development of a greater variety of species and changes in population numbers. A long period (3–9 months) of culture under variable conditions seems preferable for a more complete analysis of the aerial biota.

This preliminary investigation demonstrated that viable species of algae and Protozoa make up a portion of the aerial biota. Their importance to the study of allergens and other practical problems related to their dispersal are relatively unstudied. The species listed and similar ones often clog filters in water purification plants and foul both outdoor swimming pools and air condition units where water is employed. For these reasons alone, it seems desirable to know what species of algae are dispersed by air currents under known environmental conditions so that more effective methods for their control may be formulated.

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Occurrence of Lysergic Acid in Saprophytic Cultures of Claviceps[1]

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In 1958 Tyler (1) isolated an elymoclavine-producing strain (47A) of *Claviceps* from a sample of ergot which originally grew on *Pennisetum typhoideum* Rich. in French Equatorial Africa. It was subsequently shown (2,3) that when this strain was grown in stationary culture on a modified Stoll-Abe medium with a reduced phosphate concentration (KH_2PO_4 0.01 per cent), it accumulated a large quantity of clavine alkaloids in the culture medium. When the culture medium was chromatographed (1) an unidentified compound with an R_F value of 0.10–0.13 was noted (2,3). The compound gave a faint purple color with *p*-dimethylaminobenzaldehyde, although it did not possess the normal solubility properties of an alkaloid, and gave a negative Pauly's reaction (3). Recently, Voigt and Wichmann (4) reported that an unidentified compound with similar properties was produced in culture by their strain A.

An investigation of the behavior of mixed cultures of clavine and various peptide alkaloid-producing strains of *Claviceps* was conducted by Abou-Chaar (5). One of the peptide alkaloid-producing strains, *C. purpurea* (Fries) Tul. (2N59), was isolated from a sclerotium collected in central Nebraska in August 1959 parasitic upon *Elymus canadensis* L. Analysis of the sclerotia of this collection revealed the presence of 0.035 per cent of water-soluble alkaloids calculated as ergonovine and 0.945 per cent of water-insoluble alkaloids calculated as ergotamine. Ergotamine was shown to be the major component of the water-insoluble alkaloid fraction by paper chromatography. It was observed that when strain 2N59 was grown on the modified, low-phosphate Stoll-Abe medium with strain 47A, the concentration of the non-alkaloidal, unidentified compound in the culture medium was increased three- to four-fold. Subsequently, the following experimental work was conducted to characterize the compound responsible for the unidentified spot on the chromatogram.

Preliminary chromatographic investigations (5) revealed that the compound had properties very similar to those of lysergic acid. The suggested relationship prompted the separation of the unknown compound from the accompanying alkaloids and its further purification in order to allow a more critical study of chromatographic properties and ultraviolet absorption spectrum.

EXPERIMENTAL

Extraction and Purification of the Unknown Compound.—Ten ml of growth medium from a forty-day-old mixed culture of strains 47A and 2N59 which had grown in a diphtheria toxin culture bottle on a modified Stoll-Abe-low phosphate nutrient medium (3) were centrifuged for 30 minutes at 1750 rpm. The supernatant liquid was removed, made alkaline with 0.4 ml of diluted ammonium hydroxide solution and extracted for four minutes each with three successive portions (10, 5, and 5 ml, respectively) of ether. For quantitative purposes each ether layer was washed with three consecutive 0.5-ml portions of distilled water and the washings combined with the aqueous layer. Most of the alkaloids were removed by this extraction of the ammoniacal solution with ether.

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The extracted aqueous layer was transferred to a small beaker or evaporating dish and evaporated to dryness under reduced pressure at room temperature. During all manipulations the solution was protected from excessive exposure to light. The resulting residue was extracted with four successive portions (2, 1, 1, and 1 ml, respectively) of alcohol containing 4 per cent diluted ammonium hydroxide solution. The combined extracts were centrifuged for thirty minutes at 1750 rpm, and the clear supernatant liquid was transferred to a small beaker. The solid residue remaining in the test tube was washed with three consecutive 0.5-ml portions of alcohol, the suspension centrifuged after the addition of each portion, and the supernatant liquid added to the contents of the beaker. The combined alcoholic extract was evaporated to dryness under reduced pressure and the residue extracted with absolute alcohol using the same procedure. The resulting absolute alcohol extract was concentrated to a 0.5-ml volume under reduced pressure, streaked on a 23 cm x 57 cm sheet of Whatman No. 3 filter paper and chromatographed ascendingly by the method of Foster *et al.* (6) for lysergic acid.

The chromatogram was dried at room temperature and examined briefly under ultraviolet light. The R_F of the fluorescent band between 0.20 and 0.25 (compound A) correspond to that of lysergic acid. A fluorescent band at R_F 0.38–0.42 (compound B), which had not been noted in the preliminary chromatographic investigation, corresponded to that which has been reported for isolysergic acid (6). Strips containing compounds A and B respectively were cut from the chromatogram, and the substances were eluted separately with alcohol containing 2 per cent diluted ammonium hydroxide solution. This was found to be the best eluant for the unknown substances in that it effected complete elution more rapidly than any other solvent which was tested (chloroform, absolute ethanol, and 95 per cent ethanol). The elutions were carried out under a bell jar which was kept in the dark; a twenty-hour elution period was found to be satisfactory for qualitative studies, but the elution time was extended to forty-four hours for quantitative determinations. Each eluate was evaporated to dryness under reduced pressure, and the residue was redissolved in a total of 3 ml of absolute alcohol. The solution was centrifuged, the clear supernatant liquid was evaporated to dryness, and the resulting residue was redissolved in 3 ml of absolute alcohol. The alcoholic extract was evaporated to dryness and the residue dissolved in 0.75 ml of absolute alcohol. The ethanolic solution which was ready for chromatographic investigation was transferred to a glass-stoppered test tube and stored in a refrigerator until needed.

Chromatographic Study of the Purified Unknown Compounds.—Ten-μl portions of the absolute alcohol solutions of compounds A and B were chromatographed singly and in combination with known lysergic and isolysergic acids on 23 cm x 57 cm sheets of Whatman No. 1 paper with four different solvent systems. The water-saturated *n*-butanol system (1) employed ascending formation on paper which had been impregnated with MacIlvaine's buffer at *p*H 5.0. MacIlvaine's buffer at *p*H 7.0 was used in the procedure of Voigt (System No. 3) (7); the chromatograms were formed ascendingly with water-saturated chloroform. The system of Foster *et al.* (6) for lysergic acid involved the organic phase of *n*-butanol: pyridine:water (4:1:5) and descending formation. The *n*-butanol:acetic acid: water (4:1:5) chromatographic solvent was used descendingly.

The chromatographic behavior of the unknown compounds was also studied on buffered paper over a range of *p*H values. Strips (11 cm x 57 cm) of Whatman No. 1 paper were buffered with MacIlvaine's buffer at *p*H 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, respectively. The unknown compounds and suitable combinations with the control compounds were spotted on the sheets, and the chromatograms were formed with water-saturated *n*-butanol.

Lysergic acid had properties identical with compound A, and isolysergic acid

had properties identical with compound B. This included the same fluorescence under ultraviolet light, the same purplish-violet color reaction when sprayed with 2 per cent *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid, and comparable R_F values. The average R_F values are given in tables 1 and 2. The R_F values of the known and unknown compounds reported in table 2 for *p*H 5 are slightly higher than the values reported for the water-saturated *n*-butanol system in table 1. This may be attributed to the use of narrower sheets of filter paper, temperature variations and/or a difference in the degree of saturation of the chromatographic chamber; however, since known compounds were spotted on each chromatogram, a comparison is valid.

TABLE 1. *Average R_F values of compounds A and B and lysergic and isolysergic acids*

System	Compound A	Lysergic Acid	Lysergic Acid Plus Compound A	Compound B	Isolysergic Acid	Isolysergic Acid Plus Compound B
Water-saturated *n*-Butanol	0.12	0.12	0.12	0.29	0.29	0.29
Foster's *et al.*	0.22	0.22	0.22	0.41	0.41	0.41
n-Butanol:Acetic Acid:Water	0.55	0.55	0.55	0.56	0.56	0.56
Voigt's No. 3	0.00	0.00	0.00	0.00	0.00	0.00

TABLE 2. *Average R_F values of compounds A and B and lysergic and isolysergic acids on buffered paper formed with water-saturated n-butanol*

*p*H	Compound A	Lysergic Acid	Lysergic Acid Plus Compound A	Compound B	Isolysergic Acid	Isolysergic Acid Plus Compound B
8	0.19	0.20	0.20	0.32	0.33	0.34
7	0.11	0.11	0.11	0.31	0.31	0.31
6	0.16	0.17	0.17	0.42	0.42	0.41
5	0.17	0.17	0.17	0.40	0.41	0.42
4	0.20	0.20	0.21	0.32	0.33	0.34
3	0.23	0.23	0.22	0.23	0.23	0.23

Since thin-layer chromatography is adaptable to small samples and has some advantages over paper partition chromatography, compound A and lysergic acid were chromatographed on a thin layer of silicic acid with ethanol:ethyl acetate (8:2) by the method of Gröger.[3] This system, which is suitable for the separation and identification of chanoclavine and lysergic acid from other ergot alkaloids, did not separate compound A from lysergic acid, suggesting that these compounds are identical.

[3]Twenty-five grams of silicic acid (code no. 1169, Baker and Adamson) which had been passed through a No. 100 mesh sieve were stirred with 80 ml of a 1 per cent solution of potassium hydroxide. To this suspension were added 1.3 g. of corn starch (Argo) in 10 ml of 1 per cent potassium hydroxide solution. This mixture were heated at a temperature of 70°C for fifteen minutes, with vigorous stirring. During the heating an additional 10 to 20 ml of 1 per cent potassium hydroxide solution were added to produce a mixture which could be poured readily. The suspension was allowed to cool slightly and poured over the surface of six 9 in. x 4 in. glass plates in such a way as to form a thin, even layer. Plates prepared in this way were dried in an oven for two hours at a temperature not exceeding 75°C. They were then removed and maintained in a desiccator until used.

Ultraviolet Absorption Spectra of Compounds A and B.—Preliminary studies showed that the alcoholic eluates of compounds A and B which were used for chromatographic investigation were too concentrated and not sufficiently pure for ultraviolet absorption studies.

The absolute alcohol extracts were purified further by streaking 0.30-ml quantities of the solutions of compounds A and B on separate 23 cm x 57 cm sheets of Whatman No. 3 filter paper and chromatographing with *n*-butanol:acetic acid:water (4:1:5). A reference sample of lysergic acid was treated similarly. The fluorescent zones were eluted for seven hours with alcohol containing 2 per cent of diluted ammonium hydroxide solution and each eluate adjusted to a volume of 3.5 ml. The ultraviolet absorption spectra of the solutions of compound A, compound B, and lysergic acid were determined in a Beckman spectrophotometer, model DU. All of the spectra possessed a trough at 272.5 mμ, a broad peak at 300–310 mμ, and agreed in other respects with the published ultraviolet absorption spectrum of lysergic acid (8,9,10).

Quantitative Estimation of Lysergic and Isolysergic Acids in the Mixed-Culture Medium.—Ten ml of the culture medium of the mixed culture of 47A and 2N59 were extracted according to the outlined procedure. The two fluorescent bands which were obtained when the extract was chromatographed by the method of Foster *et al.* were eluted for forty-four hours with alcohol containing 2 per cent of diluted ammonium hydroxide solution. The eluates were combined, and the solution was evaporated to dryness under reduced pressure. Four ml of 2 per cent aqueous tartaric acid solution and 8.0 ml of Silber and Schulze's *p*-dimethylaminobenzaldehyde reagent (11) were added to the residue and stirred to a uniform mixture. Six ml of the mixture were transferred to a porcelain dish, exposed to a mercury vapor lamp (Hanovia, type 30600) at a distance of 24 cm for seven minutes, then transferred to a selected 1-cm test tube and the absorbance determined in a Bausch and Lomb Spectronic 20 spectrophotometer at 590 mμ. Transmittance was adjusted to 100 per cent with a blank composed of 2.0 ml of 2 per cent aqueous tartaric acid solution and 4.0 ml of the *p*-dimethylaminobenzaldehyde reagent. The lysergic-isolysergic acid content (mol wt 268) was calculated from a standard curve prepared with ergonovine maleate, U.S.P. Reference Standard. The assay of duplicate samples of the culture medium showed that it contained 9.0 ± 0.3 μg of lysergic-isolysergic acid per ml.

DISCUSSION AND CONCLUSIONS

The ultraviolet absorption spectra for compounds A and B, the results of chromatographic studies and the observed solubility properties led to the conclusion that compounds A and B are lysergic and isolysergic acids respectively. This is the first report of the identification of lysergic and isolysergic acids *per se* in the saprophytic culture medium of *Claviceps* species. No evidence was obtained which would indicate whether the lysergic acid and its isomer were produced by an anabolic or a catabolic pathway; however, their presence in the nutrient broth is interesting since it demonstrates the ability of the fungus to produce both the ergoline and lysergic acid nuclei in saprophytic culture. The recognized pharmacological properties of certain simple lysergic acid derivatives give further importance to this observation.

SUMMARY

1. Compounds A and B were isolated from a mixed culture of ergot strains 47A and 2N59. They were identified as lysergic and isolysergic acids respectively on the basis of ultraviolet absorption spectra, chromatographic studies and solubility properties.

2. The amount of lysergic-isolysergic acid in the broth of a mixed culture of strains 47A and 2N59 was found to be approximately 9μg/ml.

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Notes on Fungi from Assam, IV[1]

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39. **Cercospora baliospermae,** sp. n. Fig. 1

Spots irregular, 2 to 10 mm in diam, dirty white depressed with raised reddish margin, adjacent spots often coalesce and form larger spots. Stromata present. Conidiophores arise in fascicles, a few to many, pale olivaceous, paler at the tip, geniculate, septate, 34–85×3.5–4.5 μ. Conidia hyaline to sub-hyaline, cylindric to obclavato-cylindric, septate, straight, rarely slightly curved, 26–85×3–4 μ.

On the living leaves of *Baliospermum montanum*, Muell., Jagiroad, 5.ii.59, leg. S. Chowdhury. Type specimen deposited in the *Herb. Crypt. Ind. Orient.*, Indian Agricultural Research Institute, New Delhi. No. 26674.

Maculae irregulares, 2–10 mm diam, sordide albae, depressae marginibus elevatis rubescentibus, adjacentes maculae saepe coalescentes ad efformandas maculas latiores. Stromata adsunt. Conidiophori surgunt fasciculati, nonnulli ad plures, pallide olivacei, pallidiores ad apices, geniculati, septati, 34–85×3.5–4.5 μ. Conidia hyalina vel subhyalina, cylindrica vel obclavato-cylindrica, septata, recta, rarius paulum curvata, 26–85×3–4 μ.

Typus lectus in foliis vivis *Baliospermum montanum*, Muell, in loco Jagiroad, 5.ii.59, leg. S. Chowdhury, et positus in *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi. No. 26674.

40. C. CALLICARPAE Cooke in *Grevillea*, **6:** 140, 1878; Chupp, *Monogr. of the Fungus Genus Cercospora*: 588–589, 1953.
C. callicarpicola Nakata, *Mem. Coll. Agr. Kyoto. Imp. Univ.* **47:** 49, 1944.
C. callicarpicola Sawada, *Taiwan Agr. Res. Inst. Rep.* **85:** 100, 1943.

Leaf spots irregular, indistinct without any definite margin, 3–15 mm in diam, adjacent spots often coalesce forming larger spots, brown to reddish brown fading gradually into the green of the leaf. Fruiting amphigenous but more readily visible on the upper surface, scantily effuse on lower surface; small black stromata and dense fascicles on upper surface; mostly no stromata and non-fasciculate on lower surface. Conidiophores pale olivaceous brown, fairly uniform in colour except at the tip, septa not distinct, rarely or mildly geniculate, 40–125×2.5–4 μ. Conidia cylindric to obclavato-cylindric, sub-hyaline to pale olivaceous, straight to slightly curved, septate, 20–60×2.5–5 μ.

On the living leaves of *Callicarpa macrophylla* Vahl., Jangimukh, 17.viii.59, leg. S. Chowdhury. Specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi. No. 26094.

41. **Cercospora cedrelae,** sp. n. Fig. 2

Leaf spots circular to irregular, up to 12 mm in diameter, often adjacent spots coalesce and form larger blotches, dark brown; fruiting mostly hypophyllous; stromata brown, minute to 22 μ in diameter; conidiophores in fascicles, pale to medium olivaceous brown, geniculate, not branched, 18–105×3–4 μ. Conidia hyaline to sub-hyaline, obclavato-cylindric, straight to slightly curved, 22–71×2.5–3.5 μ.

On the living leaves of *Cedrela tona* Roxb., Naubaisa, 12.ii.57., leg. S. Chowd-

[1]Parts I, II and III, in Lloydia **18:** 82–87 (1955); **20:** 133–138 (1957); **21:** 152–156 (1958).

hury. Type specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi. No. 26092.

Foliorum maculae circulares vel irregulares, usque ad 12 mm diam, saepe adjacentes maculae coalescunt ad efformandas latiores maculas, fusce brunneae; fructificationes ut plurimum hypophyllae; stromata brunnea, minuta ad 22 μ diam; conidiophori fasciculati, pallide vel mediocriter olicaceo-brunnei, geniculati, non ramosi, 18–105×3–4 μ. Conidia hyalina vel subhyalina, obclavato-cylindrica, recta vel paulum curvatae, 22–71×2.5–3.5 μ.

Typus lectus in foliis vivis *Cedrela toona* Roxb., Naubaisa, 12.ii.57, leg. S. Chowdhury, et positus in *Herb. Crypt. Ind. Orient.* I.A.R.I., New Delhi. No. 26092.

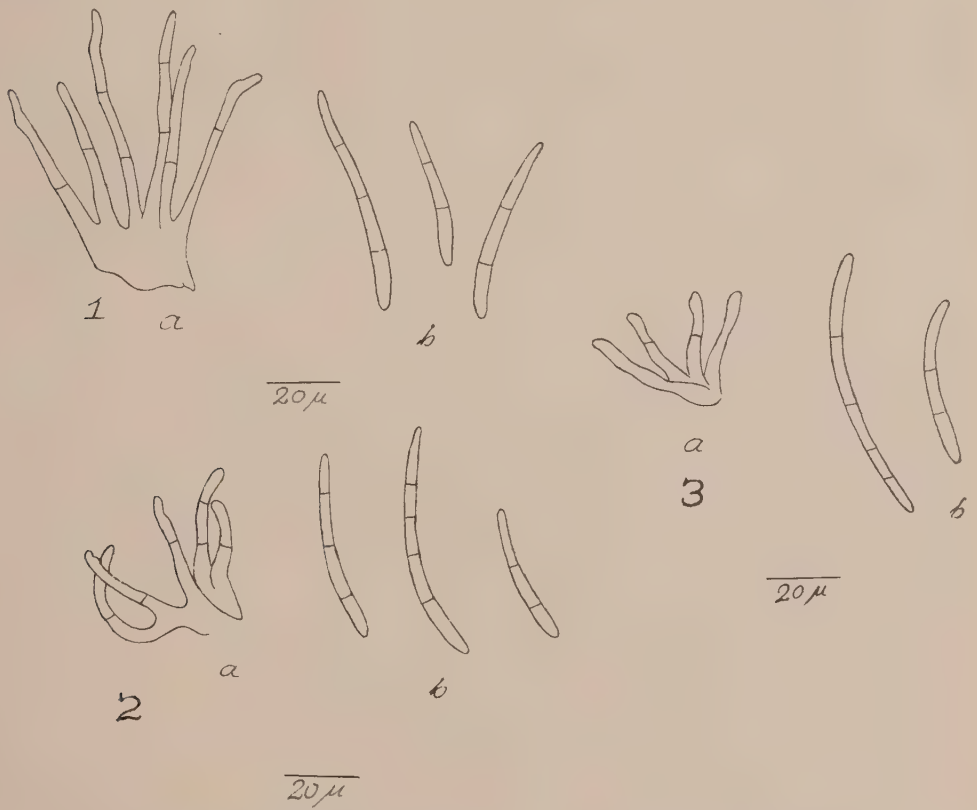

FIG. 1. **Cercospora baliospermae** *Chowdhury sp. n., a, conidiophores; b, conidia.*
FIG. 2. **Cercospora cedrelae** *Chowdhury sp n., a, conidiophores; b, conidia.*
FIG. 3. **Cercospora pogostemonae** *Chowdhury sp. n., a, conidiophores; b, conidia.*

42. C. MACARANGAE H. and P. Sydow in *Ann. Mycol.* **12:** 575, 1914; Chupp, *Monogr. of the Fungus Genus Cercospora*, 223–224, 1953.

Leaf spots spherical to slightly irregular, 2–8 mm in diam; on the upper surface grayish brown to dull grayish, sometimes with a darker margin, indistinct on the lower surface. Fruiting hypophyllous, stromata small, dark brown, fascicles dense. Conidiophores pale to medium olivaceous brown, in mass dark, paler towards the tip, not geniculate, straight, not branched, 40–170×3–4 μ. Conidia

obclavato-cylindric, sub-hyaline to pale olivaceous, nearly straight, septate, 12–80×3–5 μ.

On the living leaves of *Macaranga indica* Wight., Wahjain, 5.iii.58, leg. S. Chowdhury. Specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi. No. 26093.

43. C. MELOCHIAE P. Hennings in *Hedwigia* **43:** 395, 1904; Chupp, *Monogr. of the Fungus Genus Cercospora*, 556, 1953.

Leaf spots at first indefinite or none, later may be light brown on upper surface. Fruiting in dark olivaceous effuse patches, mostly on lower surface. Stromata lacking or small. Conidiophores pale olivaceous brown, mostly non-fasciculate, occasionally with dense fascicles, straight to curved, 10–70×3–5 μ. Conidia cylindro-clavate, pale to very pale olivaceous brown, straight to mildly curved, indistinctly septate, 30–140×2–4 μ.

On living leaves of *Melochia corchorifolia* L., Barbheta, 7.viii.59, leg. S. Chowdhury. Specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi, No. 26673.

44. **Cercospora pogostemonae,** sp. n. Fig. 3

Spots irregular, 2–15 mm in diam, often adjacent spots coalesce and form larger spots, light brown. Stromata present. Conidiophores arise in fascicles, septate, geniculate, light brown in colour, paler at the tip, 22–112×3–4 μ. Conidia sparse on the upper surface, more on the lower surface, obclavato-cylindric, straight to curved, hyaline to sub-hyaline, indistinctly septate, 48–94×3.5–4 μ.

On the living leaves of *Pogostemon villosus* Benth., Pokamura, 14.iv.59, leg. S. Chowdhury. Type specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi. No. 26676.

Maculae irregulares, 2–15 mm diam, saepe adjacentes maculae coalescentes ad efformandas maculas latiores pallide brunneae. Stromata adsunt. Conidiophori surgunt fasciculati, septati, geniculati, pallide brunnei, pallidiores ad apices, 22–112×3–4 μ. Conidia sparsa in superiore pagina foliorum, frequentiora in inferiore, obclavato-cylindrica, recta vel curvata, hyalina vel subhyalina, indistincte septata, 48–94×3.5–4 μ.

Typus lectus in foliis vivis *Pogostemon villosus* Benth., in loco Pokamura, 14.iv. 59, leg. S. Chowdhury, et positus in *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi, No. 26676.

45. C. TITHONIAE Baker and Dale in *Mycol. Papers Commonwealth Mycol. Inst.*, **33:** 106, 1951; Chupp, *Monogr. of the Fungus Genus Cercospora*, 162, 1953.

Leaf spots subcircular to irregular, 2–10 mm in diam, brown with yellowish or orange margin. Fruiting chiefly hypophyllous, stromata none or only a few brown cells. Conidiophores pale olivaceous, usually straight, not geniculate, uniform in colour and width, 26–100×3–4 μ. Conidia sub-hyaline to very pale olivaceous, obclavato-cylindric, straight or very mildly curved, occasionally catenulate, 22–60×3–4 μ.

On the living leaves of *Tithonia tagetiflora* Desf., Barbheta, 15.x.57, leg. S. Chowdhury. Specimen deposited in the *Herb. Crypt. Ind. Orient.*, I.A.R.I., New Delhi, No. 26090.

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Investigation of the Alkaloids of Paspalum Ergot

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The poisonous nature of paspalum ergot, *Claviceps paspali* Stevens and Hall, has long been recognized (1), but information as to the chemical composition of sclerotia of this fungus is not recorded in the literature. In the southern United States the organism commonly parasitizes *Paspalum dilatatum* Poir., a grass extensively grown as a forage crop. This host plant is also commonly infested with ergot in parts of Australia.

In 1939, Gieger and Barrentine (2) published a progress report of their attempts to isolate the active principle of *C. paspali*. Application of classical plant analysis procedures to a 600 pound quantity of infected paspalum seed spikes yielded an amorphous residue which did not give tests characteristic of the ergot alkaloids. No other reports of analyses of sclerotia of this fungus have appeared, but Arcamone *et al.* (3) recently reported that a strain growing on *Paspalum distichum* L. in the vicinity of Rome, Italy, was found to produce lysergic acid derivatives in submerged culture. Although quantities produced and other specific details are not presented in their preliminary communication, these authors report the isolation from the culture broth of four main compounds: lysergic acid amide, isolysergic acid amide, lysergic acid methyl carbinolamide, and isolysergic acid methyl carbinolamide.

Consideration of the economic importance of paspalum ergot as a livestock poison, coupled with the possibility of discovering new strains of *Claviceps* capable of producing lysergic acid derivatives in saprophytic culture, led the authors to undertake the present investigation. To facilitate the separation and identification of small quantities of alkaloids, a thin-layer chromatographic procedure was developed and utilized.

EXPERIMENTAL

Preparation of Thin-layer Chromatographic Plates.—For the preparation of the thin-layer chromatographic plates a number of commercially available silicic acid and aluminum oxide preparations were investigated to determine their relative suitability. Of those tested, silicic acid, code no. 1169, Baker and Adamson, (B and A quality), Allied Chemical Co., General Chemical Division, New York, proved to be the most satisfactory. "Alkaline" thin-layer chromatographic plates, which gave excellent separation of the ergot alkaloids and their derivatives, were prepared from this silicic acid according to the following procedure.

Twenty-five g of silicic acid which had been passed through a no. 100 sieve was stirred with 80 ml of a 1 per cent solution of potassium hydroxide. To this suspension was added 1.3 g of corn starch (Argo) in 10 ml of 1 per cent potassium hydroxide solution. This mixture was heated at 70° C for fifteen minutes, with vigorous stirring. During the heating an additional 10 to 20 ml of 1 per cent potassium hydroxide solution was added to produce a mixture which could be poured readily. The suspension was then allowed to cool slightly and poured over the surface of six 9 in.×4 in. glass plates in such a way as to form a thin, even layer. The prepared plates were then dried in an oven for two hours at a temperature not exceeding 75° C and maintained in a desiccator until used.

Extraction of the Alkaloids.— Two samples of paspalum ergot were investigated. The first consisted of sclerotia of *C. paspali* parasitic upon *P. dilatatum* growing

in Arkansas, USA; the second sample from the same host plant growing in Australia. Extraction of the alkaloids from the sclerotia was carried out by two different methods.

Procedure A. This is a modification of the extraction procedure of Stoll *et al.* (4). In a typical example, 4.4 g of paspalum ergot from Australia was milled to a fine powder and defatted with petroleum ether in a Soxhlet extractor for eight hours. The defatted drug was then shaken for thirty minutes with 30 ml of 70 per cent acetone containing 2 per cent of tartaric acid, and this extraction procedure was repeated twice. The extracts were combined, the acetone evaporated, and the aqueous tartaric acid solution which remained was extracted with three successive 40-ml portions of ether to remove impurities. For removal of the alkaloids, the extract was adjusted to *p*H 9 with ammonium hydroxide solution and shaken three times with successive portions of methylene chloride. Ten ml of a saturated solution of sodium chloride was then added, and the solution was again extracted with methylene chloride. All methylene chloride extracts were combined, concentrated to a small volume and chromatographed.

Procedure B. Four grams of powdered, defatted drug was extracted by shaking for one-hour periods with three successive 40-ml portions of ether to which had been added 3 drops of 10 per cent ammonium hydroxide solution. The ether extracts were united and concentrated to approximately 10 ml. This extract was then shaken out with three successive 10–ml portions of 2 per cent tartaric acid solution. The tartaric acid extracts were combined, rendered alkaline, and re-extracted with three portions of methylene chloride which were combined, concentrated and used for chromatography.

Chromatography.—The thin-layer chromatograms were formed in two different solvent systems. System I, which was especially suited for the separation of clavine alkaloids, was composed of ethyl acetate:ethanol:dimethylformamide (13:1:1). A similar system has been recommended by Klavehn and Rochelmeyer (5). In this procedure the concentration of ethyl acetate is not critical and may be varied somewhat without appreciably influencing the degree of separation achieved. System II was composed of ethanol:ethyl acetate (8:2). Time of formation of the chromatograms with either system was approximately three hours.

Additional separations of the alkaloids were carried out by paper chromatography on Whatman no. 1 filter paper with the following wash liquids:

System III—ether:acetone:water (1:2:1). The paper was previously dipped in 0.2 per cent tartaric acid solution and air-dried. Sheets (10 in.×6 in.) were formed ascendingly for approximately four hours at 16–18° C (6).

System IV—upper phase of *n*-butanol:acetic acid:water (4:1:5). Sheets (18¼ in.×22½ in.) were formed ascendingly for approximately twenty hours.

System V—*n*-butanol:acetic acid:water (40:10:7). Sheets (10 in.×6 in.) were formed ascendingly for four to five hours at 18° C.

Results.—The results obtained by chromatographing an extract of Australian paspalum ergot in systems I and II are illustrated in figures 1 and 2. It was found that on the thin-layer chromatograms, chanoclavine is separated by system I from all other clavine and peptide alkaloids tested and from lysergic acid.

For the further identification of the alkaloid spots, several extracts of the sclerotia were prepared by procedure A, streaked on thin-layer plates and formed in system I. Chanoclavine was also spotted on these plates as a reference compound. After formation, one-half of each of these plates was sprayed in the usual manner with *p*-dimethylaminobenzaldehyde (*PDAB*) reagent. The unsprayed portion of the zone corresponding to chanoclavine was scraped from each plate

and extracted repeatedly with warm absolute ethanol. These extracts were combined (solution 1), concentrated, and spotted together with reference chanoclavine both singly and in admixture on thin-layer plates and on filter paper. The thin-layer chromatograms were formed in systems I and II, the paper chromatograms in systems III and V; all were sprayed with *PDAB*.

Solution 1 gave only a single *PDAB*-positive spot in all four systems. This spot possessed the same R_F value as the reference chanoclavine and did not separate from that alkaloid when the two were cochromatographed. Typical R_F values[3] of the compound in solution 1 and reference chanoclavine were as follows:

Systems I and II—see figures 1 and 2.
System III —R_F 0.76.
System V —R_F 0.68–0.69.

The thin-layer chromatogram of the Australian paspalum ergot extract formed with system II contained a *PDAB*-positive spot just below chanoclavine whose R_F value did not correspond to any of the known ergot alkaloids tested (fig. 2). This compound remains unidentified.

Identification of the compounds responsible for the intense blue-violet fluorescent zone in the region of ergonovine when the extract was chromatographed in system I was next carried out. The fluorescent zone was scraped from the plate, extracted with warm absolute ethanol, and the extract was concentrated and rechromatographed in system I. The corresponding zone of this chromatogram was re-eluted as before and the purified solution thus obtained (solution 2) examined in chromatographic systems III and IV. Examination of the resulting chromatograms under ultraviolet light revealed two blue fluorescent spots on each which gave a blue-violet color when sprayed with *PDAB* reagent. The R_F values in both systems agreed with those of ergonovine and lysergic acid amide, which were spotted together with other known alkaloids and cochromatographed with solution 2. Mixed spots of solution 2, erogonovine, and lysergic acid amide were also chromatographed.

Although the latter two substances exhibited similar R_F values on the thin-layer chromatograms and could not be separated in systems I and II, the former system would have served to effect their initial separation from penniclavine had that alkaloid been present in the sclerotia. Penniclavine and ergonovine are not completely separable by systems III and IV, but in the absence of penniclavine these systems proved suitable for the characterization of ergonovine and lysergic acid amide. The respective compounds did not separate from ergonovine or from lysergic acid amide reference compounds which were spotted in admixture with solution 2 and cochromatographed:

R_F values in system III—ergonovine 0.62; unknown compound (Solution 2) 0.61.
lysergic acid amide 0.51; unknown compound (solution 2) 0.51.
R_F values in system IV—ergonovine 0.54; unknown compound (solution 2) 0.54–0.55.
lysergic acid amide 0.42; unknown compound (solution 2) 0.42–0.43.

A second fluorescent zone with an R_F value slightly higher than agroclavine on the thin-layer chromatogram formed with system I remains unidentified.

Three alkaloids, chanoclavine, ergonovine, and lysergic acid amide, were identified chromatographically in the sclerotia of *C. paspali* of Australian origin.

[3]These values are relative, being dependent upon such factors as the size of the chromatographic chamber, the degree of saturation, and the temperature. This is especially true for system III.

In addition to these alkaloids, two substances which exhibited properties similar to those of the ergot alkaloids were detected but remain unidentified. Since the alkaloid concentration in the investigated material was very low, further characterization of these compounds must be deferred until larger quantities of the sclerotia can be obtained.

Investigation revealed that the paspalum ergot of American origin (Arkansas) contained much smaller quantities of alkaloids than the Australian material. A 17–g quantity of the Arkansas ergot was extracted by procedure A, the extract streaked on a thin-layer chromatogram and formed in system I. One-half of the plate was sprayed in the usual way with *PDAB* reagent, revealing the presence of

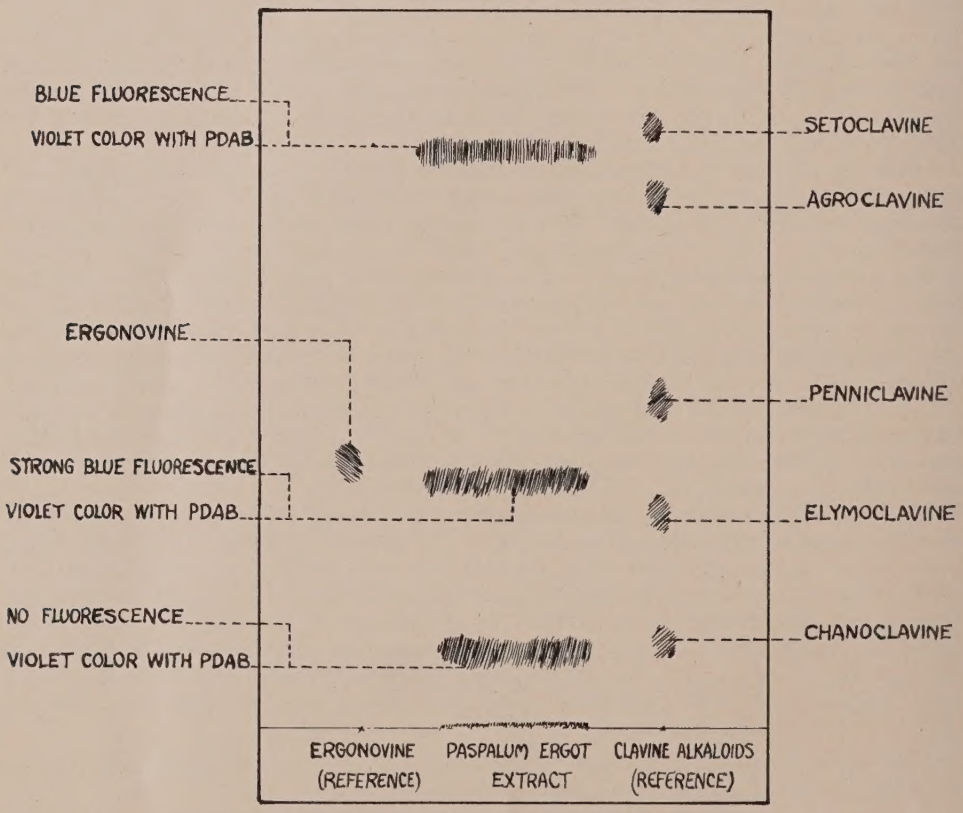

FIG. 1. *Separation of Australian paspalum ergot extract and reference alkaloids on thin-layer chromatogram formed with ethyl acetate: ethanol: N,N-dimethylformamide (13:1:1) (system I).*

chanoclavine. To verify the identity of this alkaloid the unsprayed portion of the chanoclavine spot was scraped from the plate, eluted with absolute ethanol and again spotted on a thin-layer plate. Formation was carried out with system II. Chanoclavine was also identified in this system by comparing the spot obtained from the extract with reference spots of chanoclavine which were cochromatographed. Chanoclavine was the only alkaloid which could be detected by this method in this sample of paspalum ergot (Arkansas origin).

Quantitative Determination.—Since the quantities of alkaloids in the *C. paspali* sclerotia were extremely small, a precise quantitation was not attempted. On the basis of the size and intensity of color reaction of the chromatographic spots it

was concluded that sclerotia of the *C. paspali* of Australian origin contained between 0.001 and 0.005 per cent of total alkaloids. The alkaloid concentration in the sample of American origin was one-fifth to one-tenth of this amount.

DISCUSSION

This is the first report of the occurrence of ergot alkaloids in sclerotia of *Claviceps paspali* Stevens and Hall. Lysergic acid amide has been described as a metabolic product of *C. paspali* present in the culture medium of the saprophytically cultivated organism (3), but it apparently has not been identified previously in ergot sclerotia of any species.

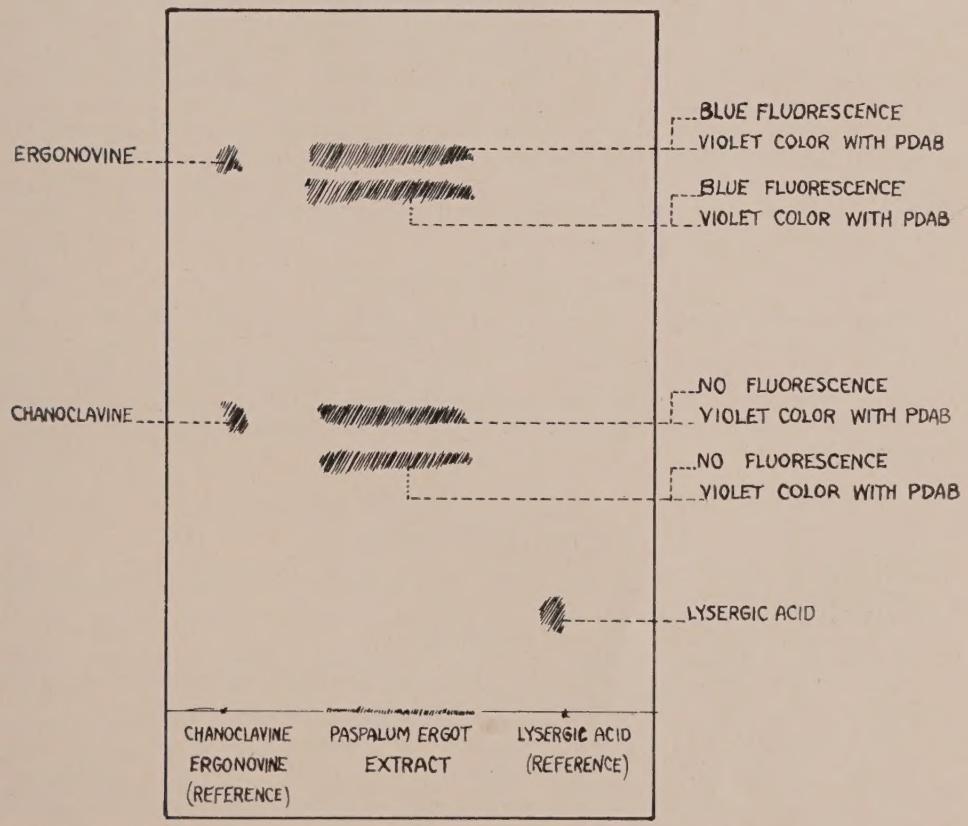

FIG. 2. *Separation of Australian paspalum ergot extract and reference alkaloids on thin-layer chromatogram formed with ethanol:ethyl acetate (8:2) (system(II).*

The distinct differences in alkaloid content of sclerotia produced on the same host plant, *Paspalum dilatatum* Poir., in Australia and in Arkansas are of considerable interest. Since much of the seed of this plant which is cultivated in the United States originates in Australia, it is tempting to speculate that observed differences may be due largely to different climatic or other environmental conditions. Such conditions no doubt play some role, but until genetically identical strains can be cultivated in both geographical areas no positive statement can be made. Certainly chemical races of *C. paspali* exist as do similar races of *C. purpurea*.

Although alkaloids were found to be present in relatively low concentrations in the sclerotia examined, the limited sampling does not permit definite conclusions to be drawn as to the role of the alkaloids in the poisoning of livestock by ergotized paspalum.

A lipid-like material which ran just behind the solvent front in all of the chromatographic systems employed and which gave an intense green color with *PDAB* reagent is worthy of further investigation as an interesting constituent of paspalum ergot. This compound was present in all crude extracts of the sclerotia which were chromatographed directly, but its relatively low water-solubility accounted for its absence in alkaloid extracts purified by partition between immiscible solvents.

Occurrence of chanoclavine in *C. paspali* sclerotia lends support to the contention that this alkaloid with its incomplete D-ring may function as an intermediate in the biosynthesis of conventional ergot alkaloids, both of the clavine and lysergic acid types (7). The presence of simple lysergic acid derivatives such as ergonovine and lysergic acid amide but the absence of peptide alkaloids in paspalum ergot indicates that the metabolism of this fungus under normal parasitic conditions is similar to that of certain other *Claviceps* species in saprophytic culture. For this reason, *C. paspali* may be looked upon as a source of strains of potential value for the production of lysergic acid or its simple derivatives in saprophytic culture, provided that the organism can be induced to produce satisfactory yields of these products.

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